1. Chapter One - General Introduction

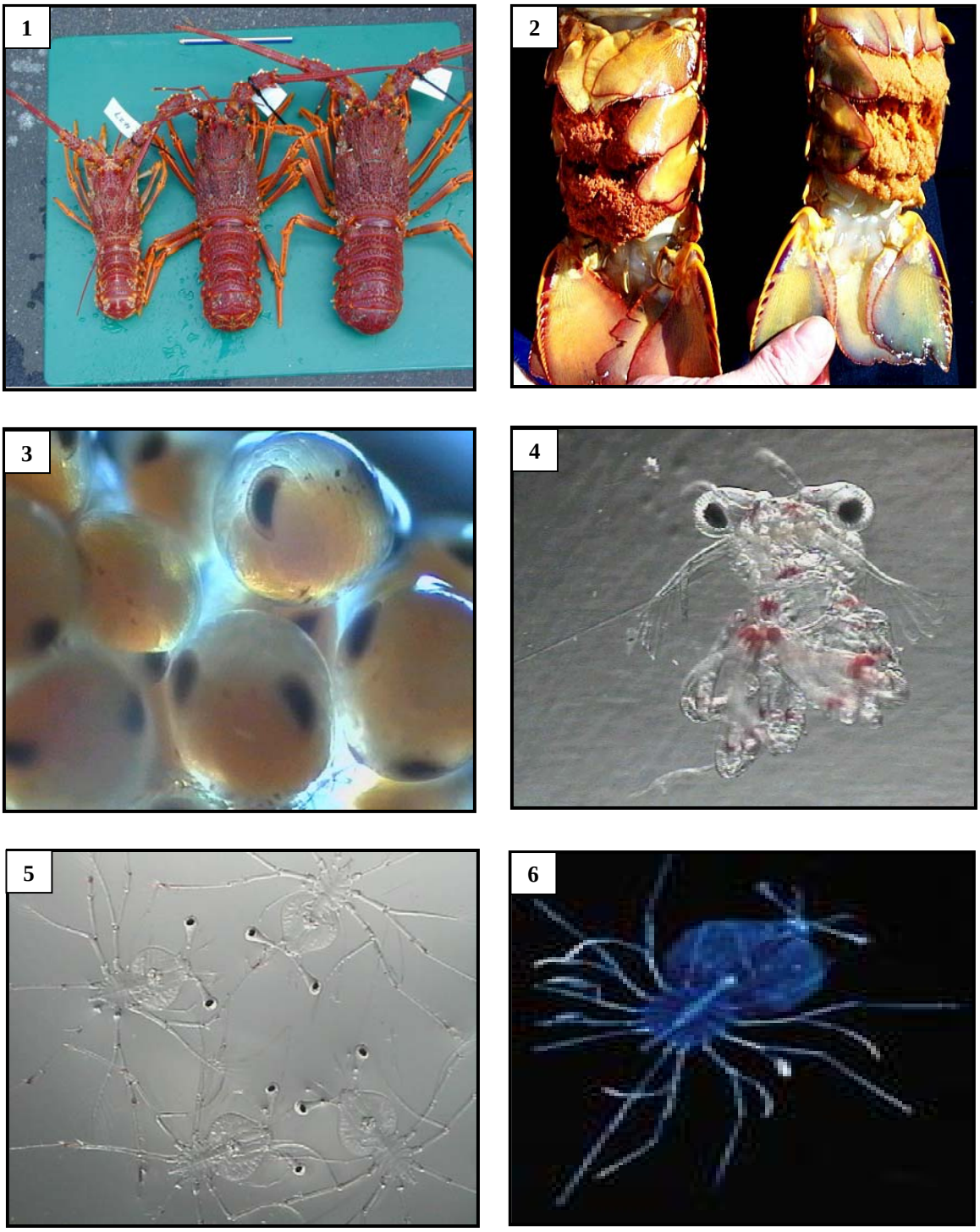

Plate 1. Developmental stages in the spiny lobster *Jasus edwardsii*, (1) broodstock, (2) egg bundles beneath ovigerous females, (3) eggs just prior to hatch, (4) newly-hatched naupliosoma, (5) Stage I phyllosoma larvae, (6) Stage VIII phyllosoma.

1.1. Introduction.

The spiny lobster *Jasus edwardsii* (Decapoda: Palinuridae) is the focus of an important crustacean fishery in southern Australia. In Tasmania, a maximum of 1500 tones per annum is set as the upper exploitation limit (Gardner et al., 2002) with expansion to lobster production only possible through the development of sustainable aquaculture (Schaap, 1997). At present, the culture of *J. edwardsii* wild-caught pueruli is being conducted in both New Zealand (Diggles, 2000) and Tasmania (D. Mills pers. com.). In New Zealand, pueruli are caught in exchange for fishery quota while in Tasmania, a percentage of the original catch of pueruli is returned after 1 year of culture. In both cases, the sentiment suggests that these are merely precursors to develop suitable culture techniques if inexpensive and efficient production of pueruli from phyllosoma larval culture becomes a reality. In Australia preliminary investigations into phyllosoma culture of a number of spiny lobster species, including *J. edwardsii*, were commenced in 1999 and to date have not achieved culture of all phyllosoma stages through to puerulus (Crear and Hart, 2001).

To provide seed stock for an aquaculture industry, reliable closure of the lifecycle must be attained. At present, this step is hindered by poor survival during phyllosoma larval culture. In *J. edwardsii* the phyllosoma larval phase encompasses 11 stages and numerous instars taking in excess of 300 days to complete in captivity (Kittaka, 1994).

At present, there is a paucity of literature on protocols required to successfully close the lifecycle of spiny lobsters. However, in recent years a number of studies have been conducted to address topics specific to *J. edwardsii* larval culture. A range of environmental requirements and tolerances have been examined focusing on light and temperature regimes (Moss et al., 1999; Tong et al., 2000; Bermudes, 2002), photoperiod, dissolved oxygen, salinity and total ammonia concentrations (Bermudes, 2002). Complementary areas of research have included the design of experimental culture systems for phyllosoma larvae (Illingworth et al., 1999; Ritar et al., 2002), the size and density requirements for phyllosoma live feeds (*Artemia*) (Ritar et al., 2003) and the lipid and fatty acid requirements of *J. edwardsii* larvae,

elucidated through starvation trials (Smith et al., 2003b; Ritar et al., in press) and the analysis of wild caught specimens (Phleger et al., 2002). Although a number of crucial areas important to the development of hatchery and feed protocols have been addressed, very little is known about larval competence in spiny lobsters. Larval competence as measured by survival and growth in culture, is often variable with total mortality occurring prior to metamorphosis to puerulus. The ability to discern, select for and enhance larval competence for experimental culture is not known. On a gross scale large differences may be evident between larvae from different females, some phyllosoma groups will be strongly photopositive while others will appear to have less vigor and settle on the tank bottom. Until the commencement of this study this was the crude basis on which larvae were selected to commence culture i.e., larvae were utilized if attracted to a light-source shortly after hatch, a technique also used to select competent larvae in penaeid hatcheries (Fast and Lester, 1992). If larval quality were associated only with swimming speed in relation to phototaxis this technique would suffice. However, it is likely that the influence of environmental, physiological and nutritional variables will have different impacts upon competency that are unable to be quantified using the current photo-response. The ability to determine larval competency in a comprehensive manner will provide a standard against which practices, both old and new may be tested, thus enhancing the prospect of successful *J. edwardsii* phyllosoma culture.

1.2. Objectives and scope of this study

1.2.1. Larval competency

Prior to the commencement of this study, experimental work using *J. edwardsii* had been conducted for a number of years in Japan (Kittaka, 1994), New Zealand (Booth, 1996) and more recently in Australia (Crear and Hart, 2001). A problem noted both in Australia (A. Ritar, pers. com.) and New Zealand (G. Moss, pers. com.) was inconsistency between survivals in larvae from different females; total

mortality was recorded periodically during an early phase of culture, often at the moult to Stage II (9-12 days after hatch). The inconvenience and associated costs involved in repeating culture experiments due to early mortalities stimulated debate as to whether initial larval competency varied as a result of parental influences, and if so, could larval competency be predicted at hatch? Therefore, one of the initial objectives of this study was to define a rapid test to predict larval competency at hatch. The development of the test for larval competency (called an activity test) is based on the loss of larval phyllosoma activity during a one-hour exposure to salinity and temperature stresses and correlation with larval phyllosoma survival in culture. The choice of salinity and temperature stressors were considered appropriate to elicit a competency response for this life-stage of *J. edwardsii,* as they have evolved to lead a predominately oceanic existence exposed to minimum fluctuations in salinity and temperature. The concept behind the activity test is that more competent larvae will have a greater ability to maintain osmotic functioning under the additional duress of elevated temperature. The scope of this study went beyond defining competence at hatch and examined a range of environmental, physiological and nutritional factors that may have an impact upon larval competence in *J. edwardsii*.

1.2.2. Environmental and physiological influences on larval competency

Manipulating water temperature to extend or shorten the duration of embryonic development to provide larvae over an extended period (Tong et al., 2000; Smith et al., 2002), a protocol also used in other crustaceans (Perkins, 1972; Branford, 1978), was examined. While this technique is very effective in extending larval availability, the impact upon larval physiology and subsequent competency was unknown. This theme was extended with an examination of lobster physiology and the effect of broodstock size on larval competency. The ability to predict the size at onset of maturity (SOM) using allometric changes in *J. edwardsii* morphology (Kubo, 1938; Gordon, 1960; Berry, 1971) were assessed in the absence of the standard indicators of maturity, such as an external egg mass or ovigerous setae (Annala et al., 1980; MacDiarmid 1989). By assessing a range of environmental and physiological influences on *J. edwardsii* broodstock standard practices may be

established to provide pre-emptive control in the selection of competent larvae for culture. An assessment of viable fecundity (number of hatched phyllosoma) compared to fishery estimates of egg number was also conducted to ascertain the need for additional studies into egg loss during embryonic development and associated broodstock husbandry procedures.

1.2.3. Enrichment protocols for phyllosoma live feeds

Artemia nauplii and metanauplii are widely used in aquaculture as live feeds for a range of fish and crustacean species (Dhont et al., 1991; Merchie et al., 1995a,b,c). However, juvenile *Artemia*, the optimum *Artemia* size for *J. edwardsii* phyllosoma (Ritar et al., 2003) are less frequently used. So while considerable literature exists on lipid and vitamin enrichment protocols for *Artemia* nauplii and metanauplii (Merchie et al. 1995 a,b,c; Evjemo et al., 1997; Estévez et al., 1998; Narciso et al., 1999) relatively less is known about juvenile *Artemia* (Olsen et al., 1999, 2000; Lim et al., 2000). To address this deficiency and to provide vital information to satisfy the dietary requirements of *J. edwardsii* larvae, dedicated lipid and vitamin studies with metanauplii and juvenile *Artemia* were conducted. Metanauplii were used as a reference to previous studies while the application of novel techniques and products helped lay the foundation for current and future studies involving juvenile *Artemia* for *J. edwardsii* and other species requiring a larger food source.

1.2.4. Nutritional influences on larval competency

The nutritional competence of *J. edwardsii* phyllosoma larvae results directly from maternal nutrition and can have a major influence upon larval viability, particularly in the provision of adequate levels of lipids and vitamins (Harrison 1990; D'Abramo 1997). As adult spiny lobsters are opportunistic foragers, the nature of this feeding strategy exposes them to a wide variety of food items, often subject to seasonal or spatial availability (Jernakoff et al. 1993; Barkai et al. 1996; Cox et al. 1997). A greater understanding of how *J. edwardsii* deals with fluctuations in diet, what organs are used as stores for particular nutritional

components, especially in relation to lipid and vitamin nutrition during ovarian maturation, may help elucidate the relative importance of dietary components to the production of competent larvae. This is of interest since viable larvae may be produced in the wild from broodstock exposed to fluctuating nutritional abundance and composition (Fielder 1965; Barkai et al. 1996). This is contrary to the view that many crustaceans require high levels of lipid and vitamin components during maturation (Cahu et al., 1984, 1994, 1995).

1.3. Notes on this study

The format of the thesis is structured with a general introduction, seven research chapters in the manuscript format of the journal Aquaculture, and concludes with a general discussion and summary. Using this style has necessitated some overlap in introduction, methods and reference sections of the research chapters. Publications are co-authored by the candidate's supervisor or co-supervisor/s in recognition of intellectual and technical contributions and the use of facilities and equipment otherwise beyond the budgetary constraints of this project. Current status of publications arising from this study is given below.

Peer Reviewed Scientific Papers

1. Smith, G.G., Ritar, A.J., Phleger, C.F., Nelson, M.M., Mooney, B., Nichols, P.D., Hart, P.R., 2002. Changes in gut content and composition of juvenile *Artemia* after oil enrichment and during starvation. Aquaculture 208, 137-158. (Chapter 4).

2. Smith, G.G., Ritar, A.J., Dunstan, G.A., 2003. An activity test to evaluate larval competency in spiny lobsters (*Jasus edwardsii*) from wild and captive ovigerous broodstock held under different environmental conditions. Aquaculture 218, 293-307. (Chapter 2).

3. Smith, G.G., Ritar, A.J., Brown, M.R., 2004. Uptake and metabolism of a particulate form of ascorbic acid by *Artemia* nauplii and juveniles. Aquaculture Nutrition 10, 1-8. (Chapter 5)

Peer Reviewed Scientific papers "In Press"

1. Smith, G.G., Ritar, A.J., Brown, M.R. Feeding juvenile *Artemia* enriched with ascorbic acid improves larval survival in the spiny lobster *Jasus edwardsii*. Aquaculture Nutrition "in press". (Chapter 8)

2. Smith, G.G., Ritar, A.J., Johnston, D., Dunstan, G.A. Influence of diet on broodstock lipid and fatty acid composition and larval competency in the spiny lobster, *Jasus edwardsii*. Aquaculture "in press". (Chapter 6)

Peer Reviewed Scientific papers "In Review"

1. Smith, G.G., Ritar, A.J. Sexual dimorphism and size at maturity in relation to fecundity and phyllosoma viability in the spiny lobster, *Jasus edwardsii*. Journal of Experimental Marine Biology and Ecology "in review". (Chapter 3)

2. Smith, G.G., Brown, M.R. Effect of dietary supplementation via *Artemia* biomass enriched with a particulate form of ascorbic acid on broodstock, egg and phyllosoma larvae of the spiny lobster, *Jasus edwardsii*. Journal of Experimental Marine Biology and Ecology "in review". (Chapter 7)

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2. Chapter Two - Lobster Competency

Published as: Smith, G.G., Ritar, A.J., Dunstan, G.A., 2003. An activity test to evaluate larval competency in spiny lobsters (*Jasus edwardsii*) from wild and captive ovigerous broodstock held under different environmental conditions. Aquaculture 218, 293-307.

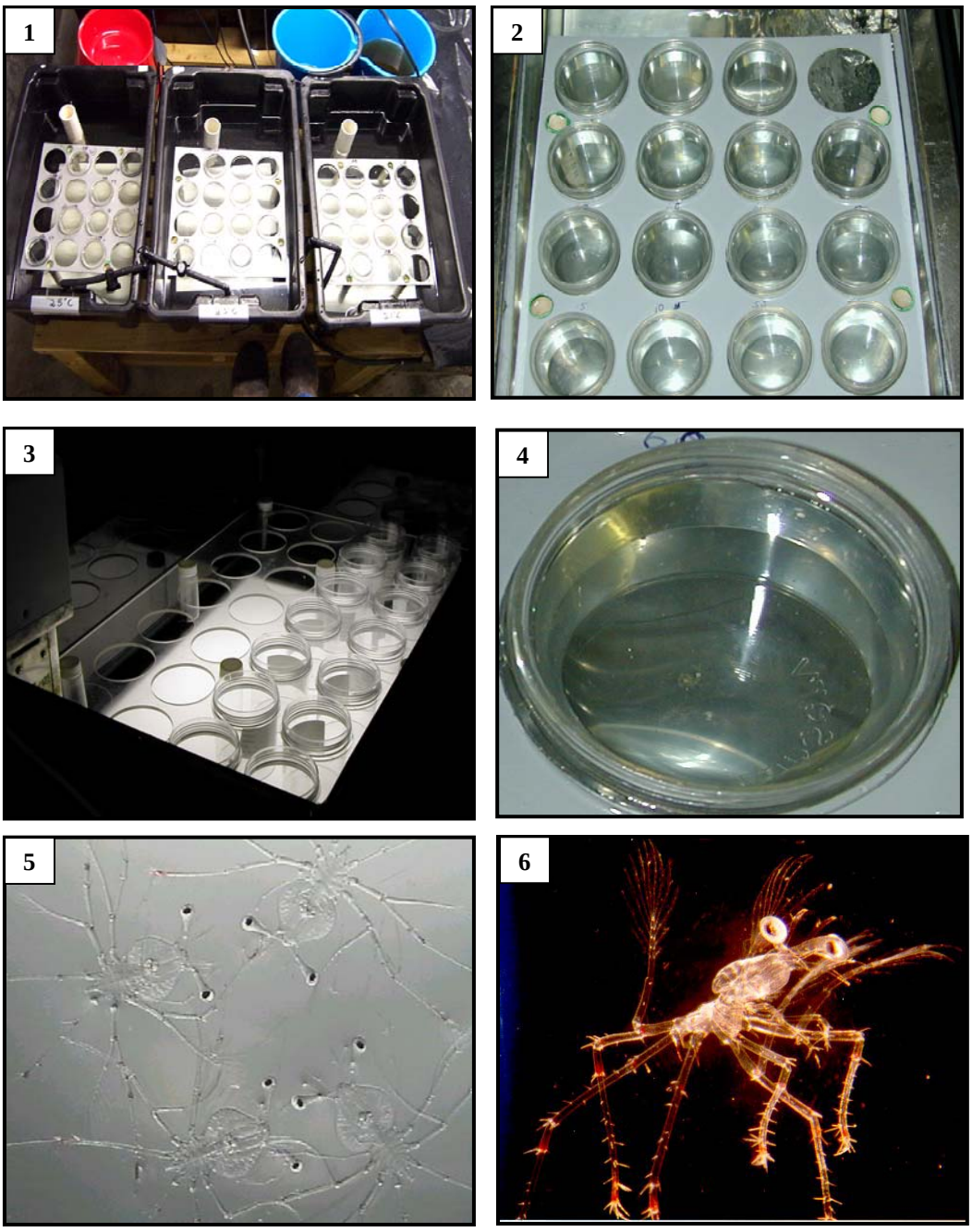

Plate 2. Development of an activity test for assessing larval competency in *Jasus edwardsii*.
(1) Initial test phase incorporating 5 salinities contained within 200 ml sample pots (in triplicate) and 3 temperatures contained in separate water baths. (2) A close-up of the sample pots in one water bath. (3) The latest version of the activity test apparatus, incorporating backlit lighting to assist in the detection of phyllosoma movement. (4) A total of 20 phyllosoma were placed within each sample pot. (5) Stage I phyllosoma larvae on the bottom of a sample pot. (6) A close-up of a Stage I phyllosoma.

2.1. Abstract

A short-term activity test was developed to ascertain the physiological condition of newly-hatched phyllosoma larvae of the spiny lobster *Jasus edwardsii* at hatch. Results of the activity test were compared with growth and survival of larvae cultured at 18°C for up to 42 days, and phyllosoma fatty acid profiles at hatch. The stresses used in the activity test comprised combinations of temperature (18, 23 and 28°C) and salinity (10, 15, 35, 55 and 60‰) applied to larvae for a 1 h period. Larvae exposed to the activity test were sourced from ovigerous captive and wild caught broodstock held at 21, 17ºC or ambient temperature (9.5 – 13.5ºC) during embryonic development. Phyllosoma originating from the 21 and 17ºC embryonic development temperatures were smaller in body length during culture compared to larvae from ambient incubated wild caught broodstock, while survival was better in larvae from ambient incubated broodstock. A strong correlation was obtained between larval activity at 23°C at 10‰ and survival of both unfed larvae cultured for 14 d (Stage I; r = 0.8720, *P* = 0.0000), and fed larvae cultured for 42 d (Stage IV; r = 0.9054, *P* = 0.0000). Elevated incubation temperature reduced the duration to hatch resulting in the quantitative sparing of a number of fatty acids. There was no correlation between activity test results and the presence of any individual or fatty acids groups in phyllosoma. The results of this study demonstrate it is possible to determine larval physiological condition using an activity test comprising temperature and salinity stresses, while additionally it was noted that larval competency is compromised when elevated temperatures are used during embryonic development to reduce the duration to hatch.

Keywords: larval competency, activity test, temperature, salinity, *Jasus edwardsii*, fatty acid.

2.2. Introduction

In Tasmanian waters, *Jasus edwardsii* females extrude eggs during declining water temperatures (May and June). Egg extrusion is followed by an incubation period lasting 5 months before phyllosoma larvae hatch during October and November providing a narrow window of opportunity in which to commence larval rearing. However, it is possible for ovigerous females to be incubated at a range of temperatures producing different rates of embryonic development and hence extending the hatch period. Phyllosoma have been produced over 5 consecutive months from August through December using this method (Tong et al., 2000; Smith et al., 2002). In a number of crustacean species elevated temperature during embryonic development accelerates the time to hatch while reduced temperatures delays hatch (Perkins, 1972; Branford, 1978; Tong et al., 2000; Smith et al., 2002). Although temperature manipulation has the ability to extend larval availability, it is not known whether larval competency (growth and survival) is also altered (Smith et al., 2002).

Another potential source of larval variability is the origin of ovigerous broodstock and whether they are obtained from wild or captive stocks. Declining broodstock competency has been noted in other species after a period in captivity possibly due to environmental factors and stress, which inhibit their ability to sequester adequate nutrient reserves prior to and during oocyte development (Palacios et al., 1998). The effects of captivity on *J. edwardsii* broodstock and their larvae have not been documented.

In many marine species, larval competency measured in terms of growth and survival only becomes statistically evident after several weeks or months of culture (Dhert et al., 1992a). As spiny lobster larval culture represents a significant investment of both time and resources, the ability to obtain a fast and accurate determination of larval competency would be desirable. The concept of instantaneously evaluating larval competency using an activity test was developed by the Japanese (Watanabe et al., 1983). It has subsequently been adapted for use in crustacean and finfish hatcheries to ascertain the susceptibility of young animals to stress (Tackaert et al., 1989; Dhert et al., 1992a,b). In formulating an activity test

for this species, we have followed a number of guidelines elicited by Tackaert et al., (1989); any test must be easy to standardize, applied and evaluated in a short time frame with simple quantification of the results possible. The parameters required to achieve this will differ between species, and indeed, between life-stages within the same species (Dhert, 1992a). In animals responsive to activity tests, a correlate can be obtained between animal activity and larval competency (Dhert et al., 1992a; Tackaert et al., 1989). At present, it is not known whether phyllosoma larvae of *J. edwardsii* are capable of such a determination. While salinity is the primary stress used in many activity tests (Tackaert et al., 1989; Dhert et al., 1992a,b), in our tests temperature was also incorporated to accentuate salinity stress. Previous experiments demonstrated that *J. edwardsii* phyllosoma have considerable tolerance to short-term salinity and temperature fluctuations (M. Bermudes, per. com.), suggesting a diverse range of temperature and salinity combinations would be required to isolate a stressor indicative of size and survival of congenetics in culture. The functional basis of activity tests may be found in a range of biochemical as well as physical larval characteristics (Dhert et al., 1992b; Rees, 1994; Abi-ayad et al., 1995; Kanazawa, 1997; Cavalli et al., 1999). A dominant biochemical factor linked to differing results in activity tests is the polyunsaturated fatty acid (PUFA) profile of the test animal and its ability to withstand osmotic stresses associated with temperature and salinity beyond its normal range. An important role of PUFAs is the maintenance of cell membrane structure and function in a variety of conditions, including during osmotic stress (Sargent, 1995).

The objective of this study was to develop an activity test comprising temperature and salinity stresses to assess the competence of newly-hatched *J. edwardsii* phyllosoma for long-term culture. Additionally the competence of larvae from wild and captive ovigerous broodstock exposed to elevated temperatures during embryonic development was examined, as was the impact of these changes on the fatty acid profile and the relationship to phyllosoma activity levels.

2.3. Materials and Methods

2.3.1. Broodstock collection and holding

Experimental work was carried out at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (TAFI, MRL), Hobart, Australia. Captive broodstock were originally caught in 1998 off the east coast of Tasmania (42° 25' S, 148° 10' E) and mated at TAFI, MRL in May 1999, whereas wild ovigerous females were caught in June 1999 off Tasmania's west coast (43° 19' S, 145° 52' E), 2-4 weeks after egg extrusion. All animals were weighed (captive - 604 ± 1.2 g; wild - 609 ± 3.1 g) and measured (carapace length; captive - 102.7 ± 0.1 mm; wild - 105.2 ± 0.1 mm) before acclimation for three weeks in a flow-through ambient system (10.5 - 9.5°C). Three different incubation temperatures were chosen, 21, 17ºC, and ambient (9.5 - 13ºC) to provide phyllosoma larvae over an extended larval rearing season.

Ovigerous broodstock were stratified on body weight and carapace length with 6 animals from both wild and captive sources randomly allocated to 4000 l square fiberglass tanks at three different incubation temperatures (total of 18 animals per broodstock source). Water supply to the ambient treatment was flow-through seawater, providing temperature gradients similar to the wild, whilst 21 and 17ºC treatments utilized a recirculation system with respective temperatures maintained using Heat‘n’chill units (Aquahort, heat pump, New Zealand). There was no noticeable difference in water quality parameters between the systems. Animals allocated to the 21 and 17ºC incubation were gradually exposed to increasing temperature over two or one-week periods, respectively, until treatment temperatures were attained. Temperatures were recorded at 11 am daily in all systems and were representative of mean daily temperatures. As a result of header buffer capacity, daily temperature variation was < 1ºC within any 24 h period, including the ambient system. Shelter was provided in each tank by 400mm lengths of 100mm PVC pipe. A 14h light: 10h dark photoperiod was maintained during embryonic development with intensity at tank floor of < 4 µmol s^{-1} m^{-2} during light

exposure. Females near hatch were placed in individual 20 l hatching containers suspended in the broodstock-hatching tank supplied with isothermal water.

At hatch, phyllosoma larvae were skimmed from the surface of the hatching container and allocated randomly to the following treatments:

- culture of larvae for 42 d with measures of total length and survival taken at regular intervals;
- survival of unfed larvae after 14 days culture;
- response of larvae to short-term temperature and salinity challenges (activity test);
- sample for analysis of fatty acids.

2.3.2. Morphological measures

Total body length (anterior tip of the cephalothorax to the posterior point of the abdomen) was measured in ten randomly chosen newly-hatched Stage I phyllosoma from each sample. Measures were again taken on days 14, 28 and 42 at Stages II, III and IV, respectively, as described by Lesser (1978). Measurements were obtained using a dissection microscope fitted with image capture (Microsoft, Vid Cap, USA) and measure software (NIH Scion image, 2000 Scion Corporation, USA).

2.3.3. Larval rearing

Larvae obtained from both sources (wild and captive) and all three incubation temperatures (21, 17ºC and ambient) were raised in static systems consisting of 1 L glass beakers immersed in individual water baths maintained at 18ºC. Initial problems with temperature regulation with individual water baths caused phyllosoma from the 21ºC wild treatment to be subjected to a high temperature spike resulting in their premature death, preventing growth and survival data being collected from this group. Water baths were thereafter connected in series and maintained at a constant temperature (18.1 ± 0.4ºC) using a Heat 'n' chill unit, and each water bath was capable of holding 6 x 1 L beakers.

Rearing for 42 d was conducted in triplicate using larvae from each female (where n = 5 females from all groups, with the exception of Stage IV captive, where n = 4). Additional triplicate groups of phyllosoma remained unfed for 14 d from hatch in the same system and under the same conditions to assess their level of endogenous reserves and physiological condition. Ecdysis, normally complete by Day 14 does not occur in unfed animals and at this stage animals were monitored for survival and treatments discontinued.

During Stage I, larval densities in both fed and unfed treatments were 100 larvae L^{-1}, larval numbers were subsequently reduced to 50 and 30 larvae L^{-1} at Stages II and III, respectively. Larval rearing was conducted in 1 L of water except when low survival in a previous stage resulted in rearing volumes being reduced to maintain constant larval rearing densities.

Phyllosoma were fed 1.5mm juvenile *Artemia* at a rate of 3 ml^{-1} every second day, and this feed coincided with total water exchange in the beakers, flushing away of uneaten *Artemia* and application of antibiotics to the culture water (Oxytetracycline hydrochloride 25ppm, Intervet Engemycin 100, Australia). On the alternate day, 1000 *Artemia* were added to each beaker to replace consumed or dead *Artemia*. Prior to use, *Artemia* were disinfected in a formalin bath (100ppm for 10 min), and rinsed on a 250 µm screen with freshwater.

2.3.4. Cumulative activity test

Newly-hatched phyllosoma (n = 20) from individual females were counted into 200 ml sample vials containing one of five salinities (10, 15, 35, 55, 60‰) in triplicate, then placed into one of three water baths (18, 23, 28°C). Phyllosoma in each vial were monitored at 3 min intervals with the number of animals prostrate on the vial floor not responding to light stimuli (halogen light, 7 µmol s^{-1} m^{-2}) with visible appendage movement counted as ‘inactive’, because at hatch phyllosoma are normally highly photopositive. Cumulative totals of inactive animals were obtained by adding the sub-totals recorded every 3 min for the 1 h duration of the test. Thus, a large cumulative total (inactive) indicated that animals had succumbed sooner to the effects of a particular temperature and salinity combination. The possible range

of results was from 0, where there were no animals inactive during the test, to 400, where all animals were inactive within 3 min and thereafter.

2.3.5. Quantification of fatty acids

Phyllosoma samples for fatty acid analysis were quantitatively extracted using a modified Bligh and Dyer (1959) one-phase methanol/chloroform/water extraction (2:1:0.8, by vol.); each sample was extracted overnight and the phases were separated the following day by addition of chloroform and water (final solvent ratio, 1:1:0.9, v/v/v, methanol/chloroform/water). The total solvent extract was concentrated (i.e. solvents removed *in vacuo*) using rotary evaporation at 40°C and lipid content determined gravimetrically. Fatty acid methyl esters (FAME) were extracted from an aliquot of the total solvent extract treated with methanol/hydrochloric acid/chloroform (10:1:1, by Vol; 80°C, 2 h) (Christie, 1982). FAME were extracted into hexane/chloroform (4:1, v/v, 3 x 1.5 ml).

Gas chromatographic (GC) analyses of FAME were performed with a Varian 3410 GC equipped with an Ultra-2 cross-linked 5% PH methyl siloxane fused silica capillary column (50 m × 0.32 mm i.d.), a flame ionization detector (FID), a split/splitless injector and a Varian 8100 auto sampler. Hydrogen was the carrier gas. Following addition of methyl tricosanoate internal standard, samples were injected at an oven temperature of 45°C. After 1 min, the oven temperature was raised to 140°C at 30°C min^{-1}, then to 310°C at 3°C min^{-1}. Peaks were quantified with Waters Millennium software (Milford, MA, USA). Comparing retention time data with those obtained for authentic and laboratory standards identified individual components.

2.4. Statistical analyses

Statistical analyses were conducted using one and two-way analysis of variance (ANOVA) with Tukey-Kramer HSD tests used for post-hoc comparison. Arcsine√ transforms were performed on percentage data (Sokal and Rohlf, 1995). Logistic regression and correlation analysis was used. Analysis of covariance (ANCOVA) was used to analyze regression slopes. Probabilities of < 0.05 were considered significantly different. Data are presented as mean ± sem. Statistics were executed using JMP version 3.2.1. (SAS Institute Inc.).

2.5. Results

2.5.1. Size of phyllosoma at hatch and during culture

Stage I larvae from ambient incubated wild broodstock were longer than animals incubated at warmer temperatures while not being significantly different to ambient incubated captive animals (ANOVA) (Fig. 2.1). Culture of phyllosoma through Stages II and III resulted in no size difference between animals incubated at 21 and 17°C. Subsequent culture to Stage IV saw ambient incubated animals from a wild source significantly larger than all other groups, which otherwise did not differ from one another. There was an effect of both broodstock source and incubation temperature (two-way ANOVA, $P < 0.05$) on the size of phyllosoma at hatch; however, incubation temperature exerted the greater influence, with no interaction occurring between these two factors. By Stage IV ambient incubated phyllosoma from a wild source had a total length 6.9% longer than ambient incubated captive phyllosoma.

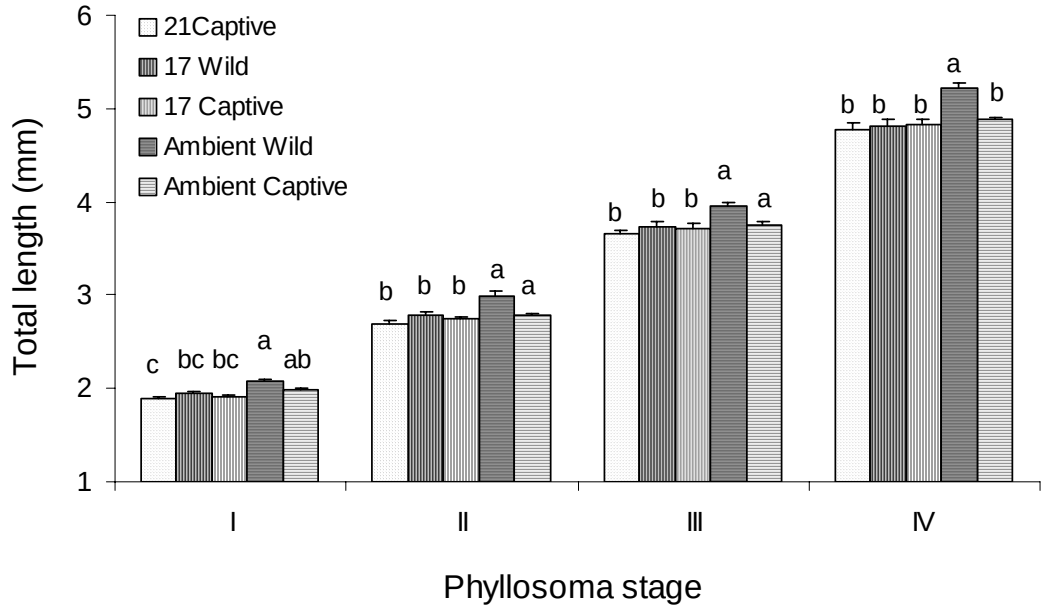

Fig. 2.1 The total length at Stages I to IV of *Jasus edwardsii* phyllosoma from different broodstock sources (wild and captive) and embryonic incubation histories (21°C, 17°C and ambient). Data are presented as mean ± sem. Different superscripts denote a significant difference between groups (ANOVA, $P<0.05$), n = 5 for all groups with the exception of Stage IV ambient captive, n = 4.

2.5.2. Survival of larvae

Incubation temperature had a significant effect on the survival of unfed 14 d old phyllosoma (two-way ANOVA, $P < 0.05$), but there was no effect of broodstock source or an interaction between temperature and source. Ambient incubated unfed 14 d old phyllosoma displayed better survival compared to unfed phyllosoma from higher incubation temperatures (Fig. 2.2).

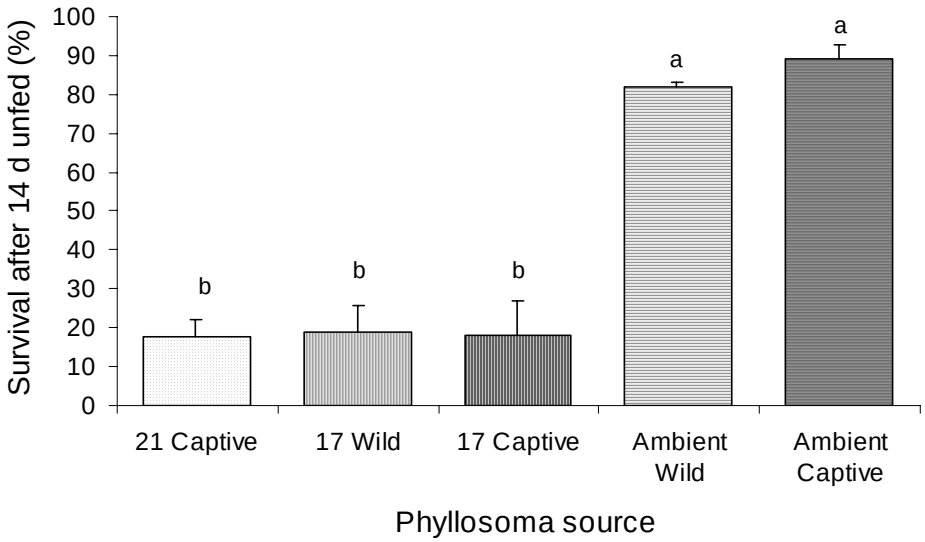

Fig. 2.2 Survival of unfed 14 d old *Jasus edwardsii* phyllosoma from different broodstock sources (wild and captive) and incubation histories (21°C, 17°C and ambient). Data are presented as mean ± sem. Different letters denote a significant difference between groups (ANOVA, $P<0.05$), n = 5.

Fed phyllosoma groups displayed no significant differences in survival at Stage II (ANOVA, $P > 0.05$) (Fig 2.3). Culture through to Stages III and IV saw significantly higher survival for phyllosoma incubated at ambient temperature than at 21 or 17°C. As was seen in unfed animals, survival at Stage IV was significantly affected by embryonic incubation temperature (two-way ANOVA, $P < 0.05$), while broodstock source and the interaction between temperature and source did not have a significant effect on the results. The regression slopes of phyllosoma survival versus developmental stage were significantly different for ambient wild and captive animals compared to all other groups (ANCOVA, $P < 0.05$), which did not differ significantly from one another (Table 2.1). A correlation between survivals in unfed 14 d old phyllosoma (Stage I) and fed phyllosoma cultured for 42 d (Stage IV) was significant ($r = 0.8656$; $P < 0.001$).

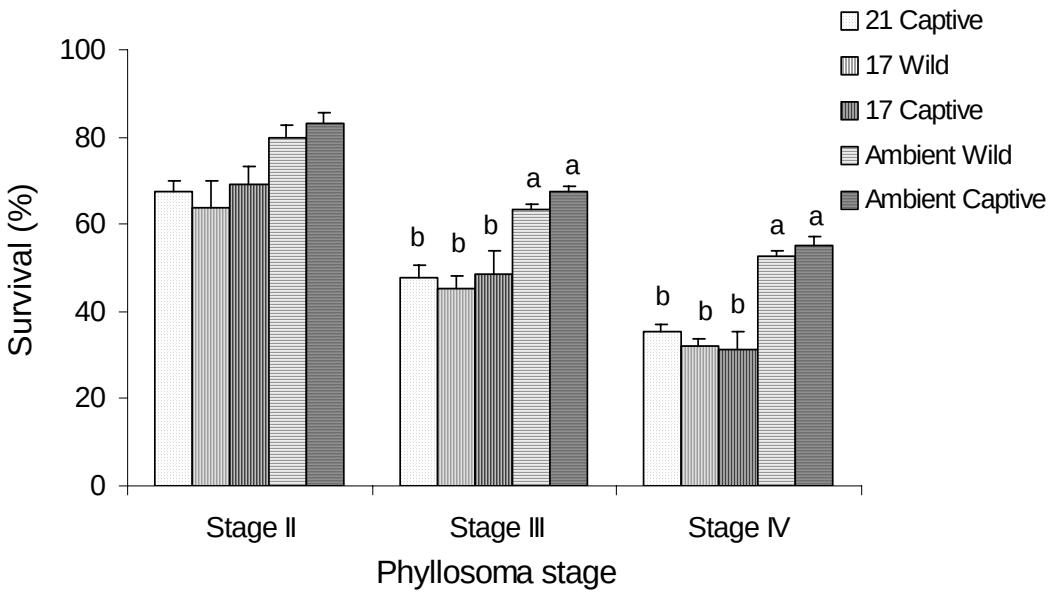

Fig. 2.3 Survival to Stage IV of *Jasus edwardsii* phyllosoma derived from different broodstock sources (wild and captive) and incubation histories (21°C, 17°C and ambient). Data are presented as mean ± sem. Different letters denote a significant difference between groups (ANOVA, $P<0.05$), n = 5 for all groups with the exception of Stage IV ambient captive, where n = 4.

Table 2.1 The regression values for a linear fit of survival of *Jasus edwardsii* phyllosoma to Stage IV against developmental Stage.

Culture groups	r^2	Prob>F	Intercept	Intercept S.E.	Slope	Slope S.E.
21 Captive	0.9315	<.0001	116.0528	3.7376	-21.3521	1.3648
17 Wild	0.8786	<.0001	115.9477	5.3449	-22.2771	1.9517
17 Captive	0.8950	<.0001	119.0517	5.0291	-22.7440	1.8364
Ambient Wild	0.9416	<.0001	113.5168	2.5465	-15.8421	0.9299
Ambient Captive	0.9624	<.0001	114.3338	1.9571	-15.1514	0.7729

2.5.3. Cumulative activity test

A total of 9 combinations of temperature (18, 23 and 28ºC) and salinity (10, 15 and 60‰) produced significantly different phyllosoma activity (i.e., the total number of inactive animals in each vial every 3 min for a 1 h duration, two-way ANOVA) (Fig. 2.4).

Correlation analysis of phyllosoma activity against survival in unfed 14 d old phyllosoma produced significant results in all but one of the same 9 activity tests, the exception being 28ºC at 60‰ (Table 2.2). The highest correlate between phyllosoma activity and survival in both unfed 14 d old phyllosoma (Stage I) and 42 d fed phyllosoma (Stage VI) occurred with the stress parameters of 23ºC at 10‰.

Table 2.2 The correlation between activity levels of *Jasus edwardsii* phyllosoma subjected to different combinations of temperature and salinity against survival for unfed 14 d old phyllosoma and 42 d old fed phyllosoma (Stage IV).

Activity test		Survival when unfed for 14 d		Survival when fed for 42 d	
Temp	Salinity	Correlation	Signif. Prob.	Correlation	Signif. Prob.
18ºC	10‰	-0.80534	0.0000	-0.64682	0.0009
18ºC	15‰	-0.58955	0.0031	-0.41599	0.0483
18ºC	60‰	0.60660	0.0136	0.47477	0.0221
23ºC	10‰	-0.87205	0.0000	-0.9054	0.0000
23ºC	15‰	-0.64710	0.0008	-0.50996	0.0129
23ºC	60‰	0.42162	0.0451	0.41802	0.0472
28ºC	10‰	-0.67866	0.0004	-0.70287	0.0002
28ºC	15‰	-0.66243	0.0006	-0.65019	0.0008

If a threshold of 50% larval survival was required at Stage IV, using the equation for the line of best fit for 23ºC at 10‰,

$$Inactivity = 455.86 - 6.68 \times Survival,$$

an activity reading smaller than 122 would be required.

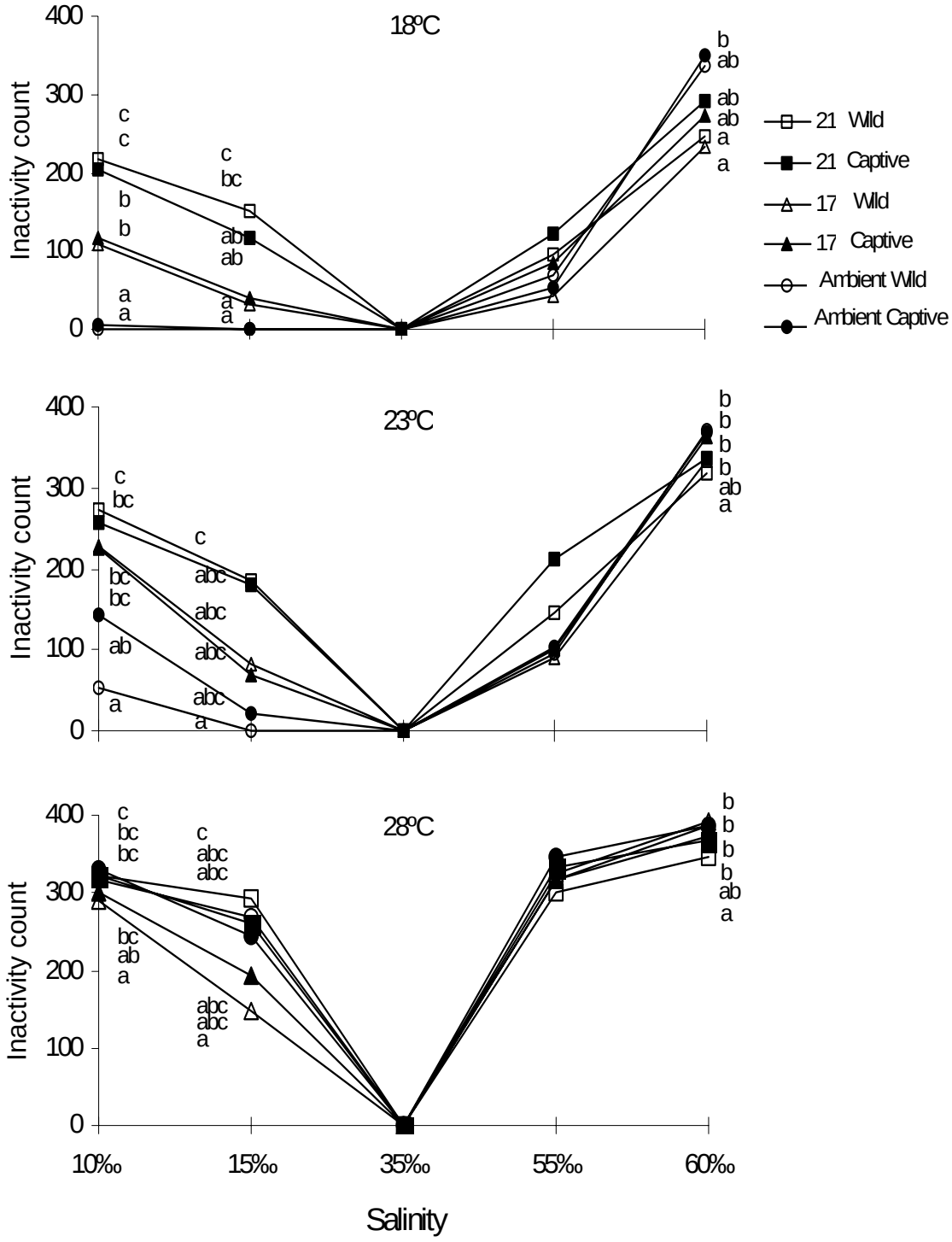

Fig. 2.4 Activity test measuring the number of prostrate newly-hatched *Jasus edwardsii* phyllosoma not responding to light stimuli at 3 min intervals for a total of 1 h when subject to different combinations of temperature (18ºC, 23ºC and 28ºC) and salinity (10, 15, 35, 55 and 60‰). Phyllosoma were from different broodstock sources (wild and captive) and incubation histories (21°C, 17°C and ambient). Data are presented as mean ± sem. Different letters denote a significant difference between groups (ANOVA, $P<0.05$), n = 5 for all groups.

2.5.4. Phyllosoma fatty acid profiles

A number of fatty acids in newly-hatched *J. edwardsii* phyllosoma were conserved at elevated (17 and 21°C) embryo incubation temperatures; in particular, the saturated fatty acids (SFA) 16:0 and 18:0, the monounsaturated fatty acids (MUFA) 16:1n – 7, 18:1n – 7 and 18:1n – 9 plus the essential fatty acid (EFA) docosahexaenoic acid (DHA 22:6n – 3) (Table 2.3). The amount of arachidonic acid (AA) within phyllosoma source (wild or captive) did not change significantly as a result of incubation temperature. However, wild phyllosoma contained more than twice the amount of AA found in captive animals. Eicosapentaenoic acid (EPA) was lowest in phyllosoma from ambient incubation temperature regardless of source. No correlation was obtained between the 23ºC at 10‰ activity test and any individual or fatty acid groups.

Table 2.3 Major fatty acids (mg g^{-1}dw) in newly-hatched *Jasus edwardsii* phyllosoma from wild and captive broodstock sources incubated at different temperatures.

Fatty acid (mg g^{-1} dw)	17 Wild	Ambient Wild	21 Captive	17 Captive	Ambient Captive
16:1n - 7	4.2 ± 0.1 [a]	2.2 ± 0.2 [b]	4.9 ± 0.7 [a]	3.7 ± 0.3[ab]	2.6 ± 0.5 [b]
16:0	15.5 ± 0.5[a]	9.7 ± 1.0 [b]	17.7 ± 1.9 [a]	14.6 ± 1.0[ab]	11.3 ± 1.7[b]
18:1n - 9c/18:3n –3[1]	14.3 ± 0.9 [a]	8.8 ± 1.0 [b]	14.7 ± 1.5	12.8 ± 0.9	10.4 ± 1.5
18:1n - 7	5.6 ± 0.2 [a]	3.8 ± 0.3 [b]	3.9 ± 0.5	3.0 ± 0.1	3.0 ± 0.3
18:0	7.8 ± 0.1 [a]	5.1 ± 0.5[b]	7.1 ± 0.7[a]	6.1 ± 0.4[ab]	4.7 ± 0.6 [b]
20:1n - 9/20:3n - 3[1]	2.6 ± 0.5	2.1 ± 0.2	4.4 ± 0.5 [a]	3.3 ± 0.2[ab]	2.5 ± 0.4[b]
20:4n – 6 (AA)	8.7 ± 0.3	*6.6 ± 0.9	2.9 ± 0.4	2.8 ± 0.1	*3.4 ± 1.0
20:5n – 3 (EPA)	16.5 ± 0.7 [a]	11.8 ± 0.8 [b]	13.9 ± 1.4	13.8 ± 0.7	11.1 ± 1.5
22:6n – 3 (DHA)	11.2 ± 0.9[a]	6.5 ± 0.6 [b]	17.0 ± 2.0 [a]	14.0 ± 1.1[ab]	9.7 ± 2.0[b]
Total fatty acids (mg g^{-1})	106.5 ± 2.8 [a]	69.6 ± 6.4 [b]	102.0 ± 11.4	85.6 ± 4.8	69.1 ± 9.2

[1]Under GC, these two components coeluted, GC-MS analysis demonstrated 18:1n-9c and 20:1n – 9 to be the predominant components.
Other fatty acids present in amounts < 2 mg g^{-1} dw included: 14:0, i15:0, 16:2n – 7, 16:1n - 5, i17:1, a17:1, 17:0, 18:3n - 6, 18:4n - 3, 18:2n - 6, 18:1n – 5, i19:0; 20:2n – 6, 20:3n – 6, 20:4n – 3, 20:1n - 7, 20:0, 21:5n – 3, 21:0, 22:4n – 6; 22:5n – 3; 22:5n – 6, 22:4n-3, 22:1n – 7 and 22:0.
Different superscripts denote a significant difference between fatty acids incubated at different temperatures within either the wild or captive broodstock source.
* Signifies significant differences between ambient incubated wild and captive animals.

2.6. Discussion

2.6.1. Size of phyllosoma at hatch and during culture

It was clear that holding broodstock at elevated temperatures increased the rate of embryonic development and resulted in smaller phyllosoma larvae at hatch. This is consistent with previous studies on larval development in *J. edwardsii* (Tong et al., 2000; Smith et al., 2002) and other crustaceans (Kunisch and Anger, 1984; Nakanishi, 1987; Shirley et al., 1987). In *J. edwardsii*, the effect of size at hatch on subsequent larval growth was not known. However, culture over several developmental stages demonstrated that larger larvae grew more at each stage than did smaller larvae. Similar differential growth has been described in other crustaceans (Kunisch and Anger, 1984; Hartnoll and Mohomedeen, 1987; Lovrich and Vinuesa, 1995) and can be attributed to increases at ecdysis being directly related to the size at the previous stage. Therefore, the effects of disproportionate growth between different size animals particularly disadvantage a species such as *J. edwardsii*, with 11 moults and numerous instars before metamorphosis to puerulus (Kittaka, 1994). In this study, we demonstrated that incubation temperature during embryonic development was the major source of larval size variation at hatch, however some of the variation was also attributed to broodstock source.

2.6.2. Survival of ongrown larvae

Broodstock that were held at elevated temperatures during embryonic development produced larvae that had lower survival during the 14 d unfed period compared to larvae from broodstock held at ambient temperature. This broodstock effect was repeated for fed larvae cultured to Stage IV. The period that larvae survive without feeding is a measure of their endogenous reserves, and in *J. edwardsii* this period is minimal, with a non-feeding “point of no return” of 4 d (Abrunhosa and Kittaka, 1997). *J. edwardsii* normally commence feeding soon after hatch with sufficient endogenous reserves sequestered after 9-12 days of

culture before undergoing a moult to Stage II. We have demonstrated in a prior study (Smith et al., 2003) that by Day 8, unfed phyllosoma larvae utilize up to 50% of endogenous lipid and body reserves. A reduction in endogenous reserves due to broodstock or incubation history would exacerbate larval physiological deficiencies and provide the basis for a positive correlation to exist between survival in 14 d-old unfed larvae and fed congenetics in culture. In findings similar to ours on larval survival, Nakanishi (1987) reported post-larval survival was negatively correlated with incubation temperature in the King crab, *Paralithodes caintschaticus*.

In some crustacean species, there is a relationship between embryonic incubation temperature, larval culture temperature and survival of larvae during culture (Diaz, 1987). In such cases, temperature acclimation during incubation provides an adaptation towards similar temperatures during culture. However, in our trial, the incubation temperatures of 21 and 17ºC did not improve survival over ambient incubated animals (9.5 – 13.5ºC) when larvae were cultured at 18ºC. This suggests that larvae experienced physiological changes due to elevated incubation temperature (small size, accelerated development) that were greater than any potential acclimation effects.

The ability of broodstock to provide the nutritional profile required for normal oocyte and larval development can profoundly affect initial larval quality (Harrison, 1990; Duray et al., 1994; Furuita et al., 2000). Broodstock sequester exogenous and or endogenous nutrient reserves prior to and during vitellogenesis to supply the nutrient requirements of their offspring. While the metabolic fate of endogenous embryonic nutrients in *J. edwardsii* is largely unknown, it has been previously demonstrated that for one essential nutrient, ascorbic acid, a positive correlation exists between nutrient conservation and low incubation temperature during embryonic development (Smith et al., 2002). It is suspected that this may also represent the fate of other essential nutrients in the current trial, contributing to a reduction in larval survival in animals incubated at elevated temperatures.

2.6.3. Cumulative activity test

It is generally agreed that when an organism undergoes alterations to their physiological state as a result of environmental or husbandry practices, that they are more susceptible to stress (Dhert et al., 1992a,b). Larvae from captive broodstock incubated outside their normal temperature range and fed a specified broodstock diet with restricted choice performed the poorest in the activity test, in terms of large inactivity counts. By contrast, ambient incubated larvae from wild broodstock with free access to a wild diet demonstrated better performance, with low inactivity counts. Performances during activity tests can be related to many physiological causes including alterations to fatty acid and lipid profiles (Dhert et al., 1992b; Abi-ayad et al., 1995; Kanazawa 1997) or environmental conditions associated with larval rearing (Dhert et al., 1992a). In the temperature-salinity combination of 23C° at 10‰, a strong relationship was established between activity levels and survival of congenetics in both 14 d unfed larvae (Stage I) and animals cultured for 42 d (Stage IV). Congenetics of phyllosoma that were immobile sooner in the activity test of 23C° at 10‰ also performed poorly in culture. The link between a specific activity test and larval competency i.e., culture performance, highlights the possibility of developing an activity test whereby a threshold survival could be assigned as the basis for deciding on whether to use the newly-hatched larvae for long-term culture. It was found that the response of larval *J. edwardsii* to a hyposaline solution best reflected their physiological condition, similar to that found in various shrimp species (Dhert et al., 1992a), whereas fish and prawns (*Macrobrachium rosenbergii*) generally elicit responses indicative of their physiological condition in hypersaline solutions (Dhert et al., 1992a).

2.6.4. Fatty acids

Lipid is a major energy source in *J. edwardsii* during egg incubation, decreasing from >40% of egg dry weight (dw) to <10% at hatch (Smith, 2000). Shorter incubation periods resulted in fatty acid conservation and thus potential energy savings, but this did not translate into improved survival in either unfed or Stage IV

fed animals during culture. It is likely that physical changes to early hatching phyllosoma had a greater impact on survival than supplementary lipid reserves, and negates any correlation that may have existed between individual or fatty acid groups and activity test results. The pattern of metabolism of individual fatty acids in relation to incubation temperature confirmed prior results for starved and fed animals (Smith et al., 2003). The reduction in monounsaturated fatty acids in this species is linked to their use as a major energy source, as is the case in many other species (Castell, 1982; Vasquez, et al., 1994; Querijero, et al., 1997), while roles for 16:0 and 18:0 have been suggested in supporting metabolic functioning rather than as a direct energy source (Smith et al., 2003). DHA does not appear to be required in excess of EPA in *J. edwardsii*, at least at this stage of development, as is often the situation in other marine species (Sargent, 1995). Sparing of AA in this trial across both wild and captive animals suggest a moderate requirement for this PUFA, particularly as AA is involved in a number of biochemical pathways including the production of eicosanoids. Eicosanoids are highly biologically active molecules, which are important for moulting, growth and the stress response (Lytle et al., 1990; D'Souza and Loneragan, 1999). While poor broodstock nutrition can have an impact on larval size (Duray et al., 1994) in this case it cannot be related specifically to fatty acid nutrition.

2.7. Conclusion

It was confirmed that elevated incubation temperature during embryonic development produced smaller larvae at hatch that performed worse in terms of survival during culture in both unfed and fed treatments. A positive correlation was obtained between survival in 14 d (Stage I) unfed larvae and animals cultured to 42 d (Stage IV), revealing that survival in short-term starvation trials are a measure of the larval competence of congenetics in culture. Positive correlations were obtained between larval activity levels in 23°C at 10‰ and survival in both 14 d unfed larvae (Stage I) and larvae cultured for 42 d (Stage IV) indicating that *J. edwardsii* are amenable to the development of an activity test for the assessment of larval

competency. However, further refinement of the temperature/salinity parameters for the activity test may be required to discern differences between 'normal' larvae obtained from broodstock held under ambient conditions during embryonic development. Fatty acids were conserved in phyllosoma with shorter embryo incubation duration however, there was no significant correlation between activity test results and levels of individual or fatty acid groups.

2.8. Acknowledgments

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3. Chapter Three - Broodstock Characteristics

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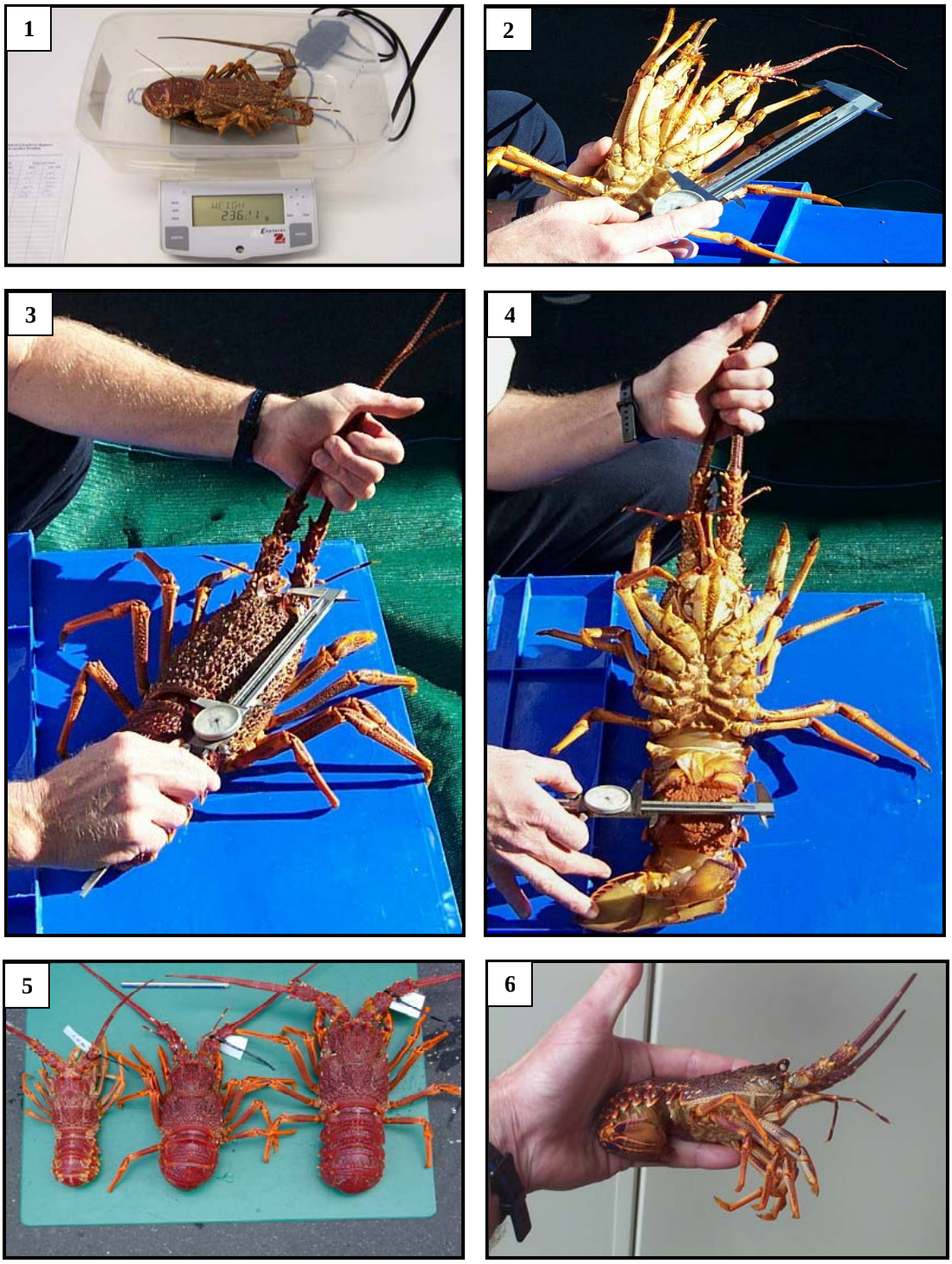

Plate 3. Physical measures of *Jasus edwardsii* spiny lobsters - (1) total wet weight (ww), (2) leg length, (3) carapace length (CL), (4) abdominal segment width. A range of lobster sizes were used in fecundity measures (5) in this photo animals ranged from smallest at 107mm CL, 600 g ww; medium at 120mm CL, 900 g ww and the largest measuring 152mm CL, 1800 g ww. (6) Small precocious ovigerous female measuring 58 mm CL, 103 g ww.

3.1. Abstract

Reproductive and somatic parameters of captive juvenile and adult *Jasus edwardsii* were examined in terms of size at onset of maturity (SOM) and fecundity, culminating in an examination of how adult size may relate to larval competency. The SOM was much smaller in captive animals (62.5mm carapace length, CL) compared to historic fishery's data (93.2mm CL) and indicated that precocious maturation may be induced in captivity. Fecundity comparisons were obtained using historical records of the wild fishery based on egg number, while a more pertinent figure for aquaculture may be viable fecundity i.e., the number of viable phyllosoma produced. During this study, we saw that viable phyllosoma number was ≈ 45% of fishery egg estimates, suggesting either declining egg numbers in wild stocks over time or that major losses in viability or egg number can occur during incubation. The association between SOM and sexual dimorphism was examined using measures of carapace length (CL), total length, length and width of the 1st and 2nd abdominal segments, and length of the 2nd and 3rd walking legs. The determination of SOM through morphometric measures, previously demonstrated in other spiny lobster species, was established for *J. edwardsii*. In females the SOM was concomitant with increases to the width of the 1st and 2nd abdominal segments above 62.5 mm CL, while 2nd and 3rd leg length increased disproportionately in males compared to females above 77.5 mm CL. The result of this study suggests that large females produce larger, more competent larvae. There were significant correlations between viable fecundity and female size (r = 0.9202), phyllosoma size (r = 0.7370) and larval viability as quantified by stress indices (r = -0.5612) and LD–50 (r = 0.5626), indicating that larger females produce larger, more viable larvae. Viable fecundity was less than half that suggested from fishery data with considerable plasticity also noted in the SOM. These are physiological traits capable of having a large impact upon fishery and aquaculture production and highlight the need for dedicated fishery and aquaculture research to be conducted in these areas.

Keywords: fecundity, *Jasus edwardsii*, larval quality, morphometrics, sexual maturation.

3.2. Introduction

The spiny lobster *Jasus edwardsii* is endemic to southern Australian and New Zealand waters. There is an important fishery for this species located in Tasmania, which currently supports an annual catch of 1500 tonnes per annum (Gardner et al., 2002). A major determinant of the sustainability of spiny lobster fisheries is their lifetime egg production (E/R) and comprises factors that include size at onset of maturity (SOM) (Wenner, 1974; MacDiarmid, 1989; Pollock, 1991, 1997) and size-specific reproductive output (fecundity).

SOM can vary considerably in spiny lobsters; it is often locality-specific and controlled by phenotypic expression (Pollock, 1997). The SOM of a population is noted as the size when at least 50% of animals in a 5 mm CL size class are mature (George and Morgan, 1979). Influences on SOM may include environmental, biological or social factors involving specific interactions between temperature, food, shelter, growth rate, age, metabolic rate and population density (Annala et al., 1980; Pollock, 1993), larger SOM results in larger E/R (Pollock, 1993). Sexual maturity in *J. edwardsii* females can be assessed by noting the presence of eggs attached to pleopods, or in the absence of eggs, well-developed ovigerous setae (Annala et al., 1980; MacDiarmid, 1989). The onset of sexual maturity in spiny lobsters has a number of morphometric ramifications, which were first noted by Kubo (1938) investigating claims that two spiny lobster variants occupied the same habitat in Japanese waters. Their morphometric study revealed that animals considered as separate forms of a similar species were the two sexes of *Panulirus japonicus*. A prominent trait recognized by Kubo (1938) was the allometric elongation of male legs and hence sexual dimorphism based on the relationship between leg length and sex. Since then, the interactions between the morphology of male and female spiny lobsters and SOM have been studied in several species and extended to incorporate female tail characteristics (Gordon, 1960; Berry, 1971). The generalized pattern in spiny lobsters is for females to have broader tails than

males to facilitate egg carrying, while males develop a larger carapace and legs, which play a role in courtship and mating (Frusher et al., 1999), it is suspected similar traits exist in *J. edwardsii*.

Fecundity measures in crustaceans are generally expressed in terms of egg number and are based on an arithmetic relationship with carapace length (CL) (Herrick, 1895), hence the maximum number of eggs is constrained by body size (MacDiarmid, 1989). While increasing E/R may be advantageous to larval production in wild populations, large SOM in culture means that energy reserves are partitioned to somatic rather than gonadal growth.

From an aquaculture perspective, it is not known how viable fecundity i.e., the number of newly-hatched phyllosoma produced by a female, relates to fishery estimates of egg production particularly as captive animals are subject to husbandry stresses during incubation. In Australian waters, egg loss in *J. edwardsii* is considered to be low (Morgan, 1972; Kennedy, 1993 unpublished Tasmanian fishery data) although there are reports of significant losses in crustacean species in other waters (Perkins, 1971; Annala and Bycroft, 1987; Waddy and Aiken, 1991; Tuck et al., 2000). Thus, there may be a need to review broodstock husbandry procedures if it is considered that they reduce viable fecundity or adversely affect larval viability.

In this study, we examined the timing of SOM and whether there were subsequent changes to lobster allometry associated with its onset, the relationship between viable fecundity and egg numbers as reported in the literature and whether viable fecundity was associated with egg or phyllosoma size and larval survival.

3.3. Materials and Methods

3.3.1. Broodstock collection and holding

Experimental work was carried out at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (TAFI, MRL), Hobart, Australia. A total of 238 juvenile and adult *J. edwardsii* involved in the study were sourced from

Flinders Island (40° 32' S, 148° 16' E), the Bicheno region (41° 51' S, 148° 21' E) and Crayfish Point (42° 56' S, 147° 20' E) between June 1999 and June 2000 (Fig. 1). The distribution of gender and CL, from the different sources were; Flinders Island, 21 males (105.5 – 116.0mm) and 39 females (101.6 – 154.0mm); Crayfish Point, 25 males (132.2 – 170.0mm); captive stock collected from Bicheno 8 adult males (115 – 127.2) and 27 adult females (106.6 – 151.1mm), and those collected as puerulus 2 years prior to the study; 62 males (29.9 –93.3mm) and 56 females (30.0 – 87.2mm). Animals collected as puerulus and held in captivity for 2 years were used as a discreet group to determine SOM in captivity. Analysis of covariance determined there was no significant difference between the relationship of CL and animal morphometrics (total length, abdominal segment length and width and leg length) attributed to source, it was therefore considered appropriate to pool source data in the comparison of male and female morphometrics.

Post-capture, all lobsters were held in 4000 L square fibreglass tanks, with adults at a density of 30 – 40 $tank^{-1}$ while pueruli/juveniles were held at 150 - 200 $tank^{-1}$. Tanks were supplied with flow-through seawater at 600 l h^{-1} under conditions of ambient temperature (seasonal range 9.5– 18.5°C) and photoperiod (seasonal range 8.5-15.3 h daylight supplied at < 4 µmol s^{-1} m^{-2}). Tanks contained a series of concrete blocks arranged to provide numerous lobster shelters. Animals were fed a diet of mussels (*Mytilus edulis*), squid (*Nototodarus sloanii*) and penaeid pellets (Vital prawn, Higashimaru-Japan) 3 times $week^{-1}$ to satiation.

3.3.2. Size at onset of maturity

SOM was determined for captive stock originally sourced from the Bicheno region, it was assessed as the midpoint of the smallest 5-mm CL size class where at least 50% of animals were sexually mature (Annala et al., 1980; MacDiarmid, 1989). As sampling was conducted during the latter part of the egg-bearing season (August, mid-winter), animals were considered mature if they were bearing external eggs (Annala et al., 1980). The monitoring of ovigerous setae on the endopodites of the pleopods (Kensler, 1967; Annala et al., 1980; MacDiarmid, 1989; Groeneveld and Melville-Smith, 1994) was deemed unnecessary with none present on non-

ovigerous animals < 60 mm CL, while 90-100% of animals >60 mm CL were ovigerous.

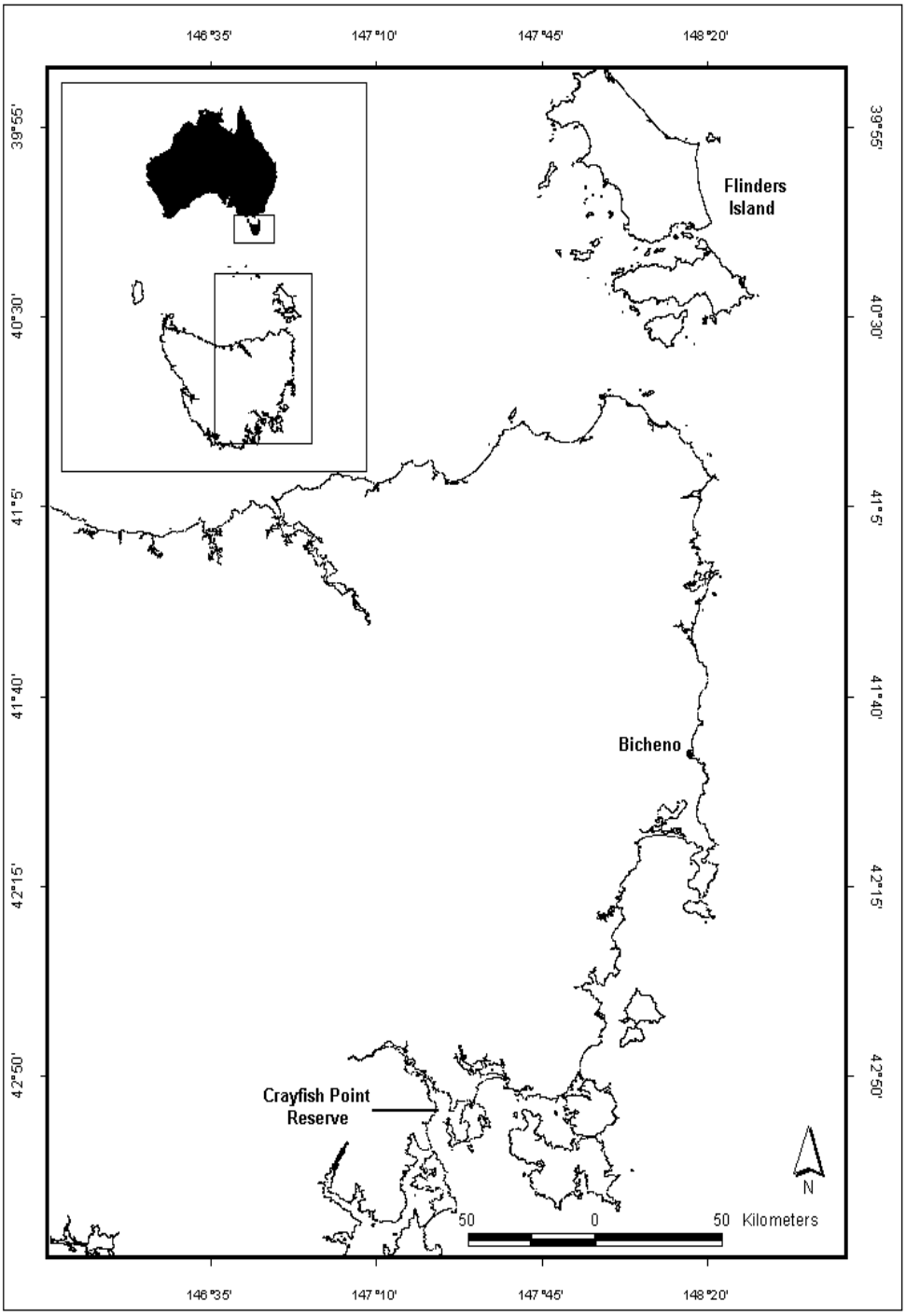

Fig. 3.1 Map of east coast of Tasmania showing the source of animals used in this study.

3.3.3. Broodstock morphological measures

Morphological measures conducted on *J. edwardsii* spiny lobsters after the completion of larval hatch period included;

- Wet weight (ww) - body weight of the animal when removed from the water for 30 s to allow the draining of excess water.
- CL - the distance from the base of the antennal platform to the dorsal posterior margin of the carapace along the dorsal midline.
- Total length - the distance from the base of the antennal platform to the tip of the telson measured along the dorsal midline.
- Abdominal segment length - the longitudinal distance between the extremities of the segment cuticle measured along the dorsal midline.
- Abdominal segment width - the maximum distance between the pleural spurs.
- Leg length - the distance measured along the ventral midline of the leg from the proximal margin of the ischium to the dactyl tip.

All broodstock morphological measures were conducted to the nearest 0.1 mm while weights were recorded to the nearest 0.5 g. The presence of a relationship between CL and total length, the length and width of the 1st and 2nd abdominal segments and the length of the 2nd and 3rd legs were examined in males and females.

3.3.4. Collection and counting of viable phyllosoma

Viable fecundity was the total of all phyllosoma resulting from each individual female and does not include egg loss, infertile or non-viable eggs or larvae failing to progress beyond naupliosoma. Naupliosoma are a brief non-feeding post-hatch stage typically lasting 0.5 - 1 h before the moult to Stage I phyllosoma (Tong et al., 2000). Proximity to hatch was determined by change in egg colour (from red at egg extrusion becoming orange and finally translucent brown at hatch) and monitoring of egg eye indices (Tong et al., 2000). About one week prior to hatch, ovigerous females were isolated into individual 20 L hatching containers (Smith et al., 2003a). Larvae hatched synchronously at dawn on each of 4 – 7 consecutive days (Smith et al., 2002) were then transferred to 10 L of aerated 18°C seawater and the number

estimated from triplicate 75 ml subsample counts. Cumulative totals of phyllosoma for each female were tallied over the duration of the hatch-out period.

3.3.5. Phyllosoma viability and morphological measures

Newly-hatched phyllosoma were subjected to a number of treatments, including;

- Lethal Dose 50 (LD–50), the time (d) for 50% of unfed Stage I larvae to die. This is a measure of endogenous reserves and physiological condition. Larvae (100 L^{-1}) were cultured in triplicate 1 L glass beakers containing lightly aerated seawater (35‰, 18°C) and antibiotics (Oxytetracycline hydrochloride 25ppm, Intervet Engemycin 100, Australia). Water was exchanged and larvae counted daily. Treatments were terminated upon the attainment of an average 50% mortality across replicates.
- A 1 h temperature/salinity activity test (Smith et al., 2003b). Briefly, triplicate 250 ml plastic sample jars containing 20 larvae were exposed to stressors of warm water (21°C) and low salinity (10‰). Survival was monitored at 3 min intervals, with a cumulative stress index compiled at the completion of 1 h exposure. Low indices are indicative of better survival in culture.
- Measures of egg and phyllosoma size. A total of 20 eggs and phyllosoma were measured on the first day that phyllosoma began to hatch from a female. Cross-sectional diameters of eggs and the total lengths of phyllosoma (anterior tip of the cephalothorax to the posterior point of the abdomen) were measured to the nearest 0.05 mm using an overhead projection microscope (Nikon Profile Projector, model 6C, 20 times magnification).

3.4. Statistical analyses

Statistical analyses were conducted using one-way analysis of variance (ANOVA) with Tukey-Kramer HSD tests used for post-hoc comparison (Sokal and Rohlf, 1995). Log transforms were performed on non-homogeneous data. In the analysis of sexual dimorphism, the point of differentiation was determined by dividing the parameter (leg length, carapace length or width) into 5 mm CL size classes (i.e., 55-59.9 mm, 60-64.9 mm, etc.) for comparison. When consecutive size

classes demonstrated sexual dimorphism, the midpoint of the smallest class was nominated as the point of deviation. Probabilities of < 0.05 were considered significantly different unless stated otherwise. Data are presented as mean ± sem. Statistics were executed using JMP version 5 (SAS Institute Inc., Cary, NC, USA.).

3.5. Results

3.5.1. Lobster morphometrics

Animals utilized for the morphometric study ranged in size from 29.9 – 170.0 mm CL for males and 30.0 – 154.0 mm CL for females. A number of other parameters were recorded including wet weight, 1st abdominal segment length and width, 2nd abdominal segment length and width, 2nd and 3rd leg length (Table 3.1).

Table 3.1 Minimum and maximum measurements of the spiny lobster *J. edwardsii*.

Parameter measured	Male (n=116)		Female (n=122)	
	Min	Max	Min	Max
Carapace length (mm)	29.9	170.0	30.0	154.0
Total length (mm)	74.0	415.0	73.5	404.0
Wet weight (g)	11.5	2220.0	13.0	1766.0
1st abdominal segment length	7.2	42.2	8.3	39.4
1st abdominal segment width	15.0	84.0	16.1	97.2
2nd abdominal segment length	7.2	43.3	8.3	41.0
2nd abdominal segment width	14.4	83.8	16.1	99.0
2nd leg length	34.0	255.0	35.0	199.0
3rd leg length	36.0	283.0	40.0	220.0

Analysis of morphometrics revealed the interaction between CL and lobster ww which was best described by the power equation $y = a*CL^b$, where ‘a’ and ‘b’ are constants with lobster ww increasing at a greater rate than CL but the relationship was similar for both sexes (Fig. 3.2).

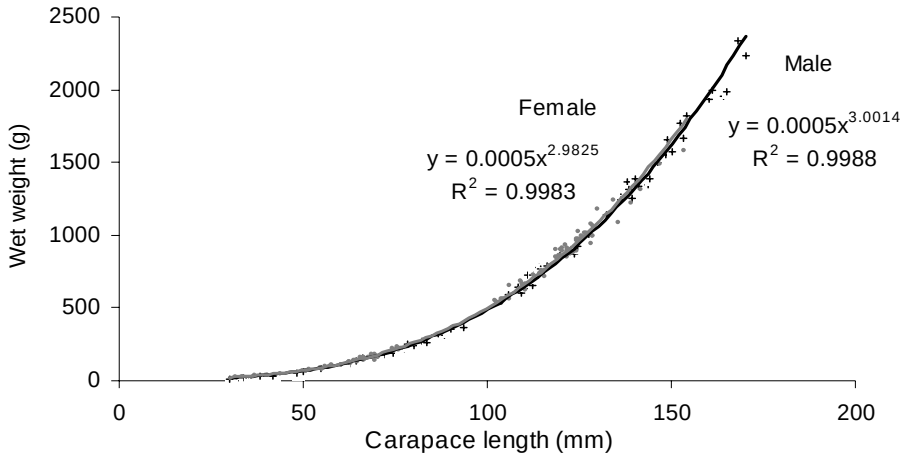

Fig. 3.2 The relationship between carapace length (mm) of male (n = 116) and female (n = 122) *J. edwardsii* and wet weight (g).

Linear relationships existed between CL and total length (Fig. 3.3) but differing significantly between sexes ($F_{1,232} = 84.31$, $P<0.0001$). CL was proportionally longer in males than females when compared to total length.

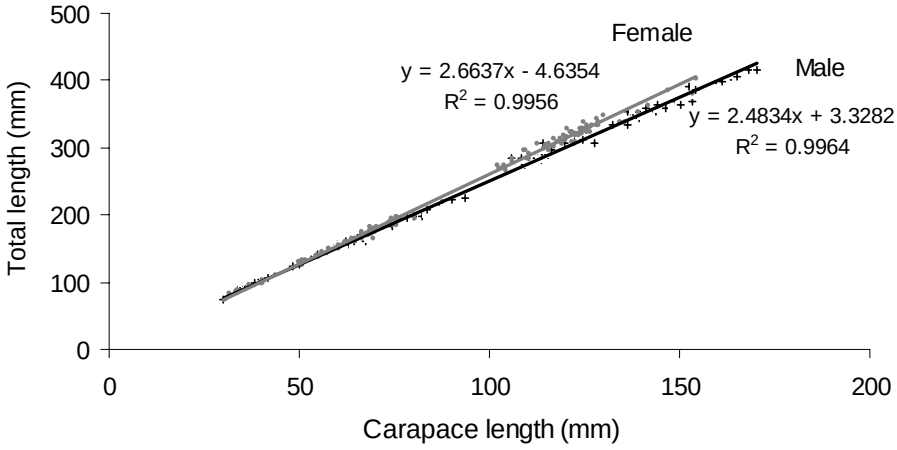

Fig. 3.3 The relationship between carapace length (mm) of male (n = 116) and female (n = 122) *J. edwardsii* and total length (mm).

Regression analysis of CL and the length of the 1st abdominal segment demonstrated significant differences between males and females ($F_{1,232} = 18.06$, $P<0.0001$, Fig. 3.4a) with females having longer abdominal segments than males. Similar results were obtained when analyses were extended to the 2nd abdominal

segment ($F_{1,232}$ = 24.90, P<0.0001, Fig. 3.4b). However, analysis of the 5 mm CL size classes could not discern the point where the length of the abdominal segment deviated (ANOVA).

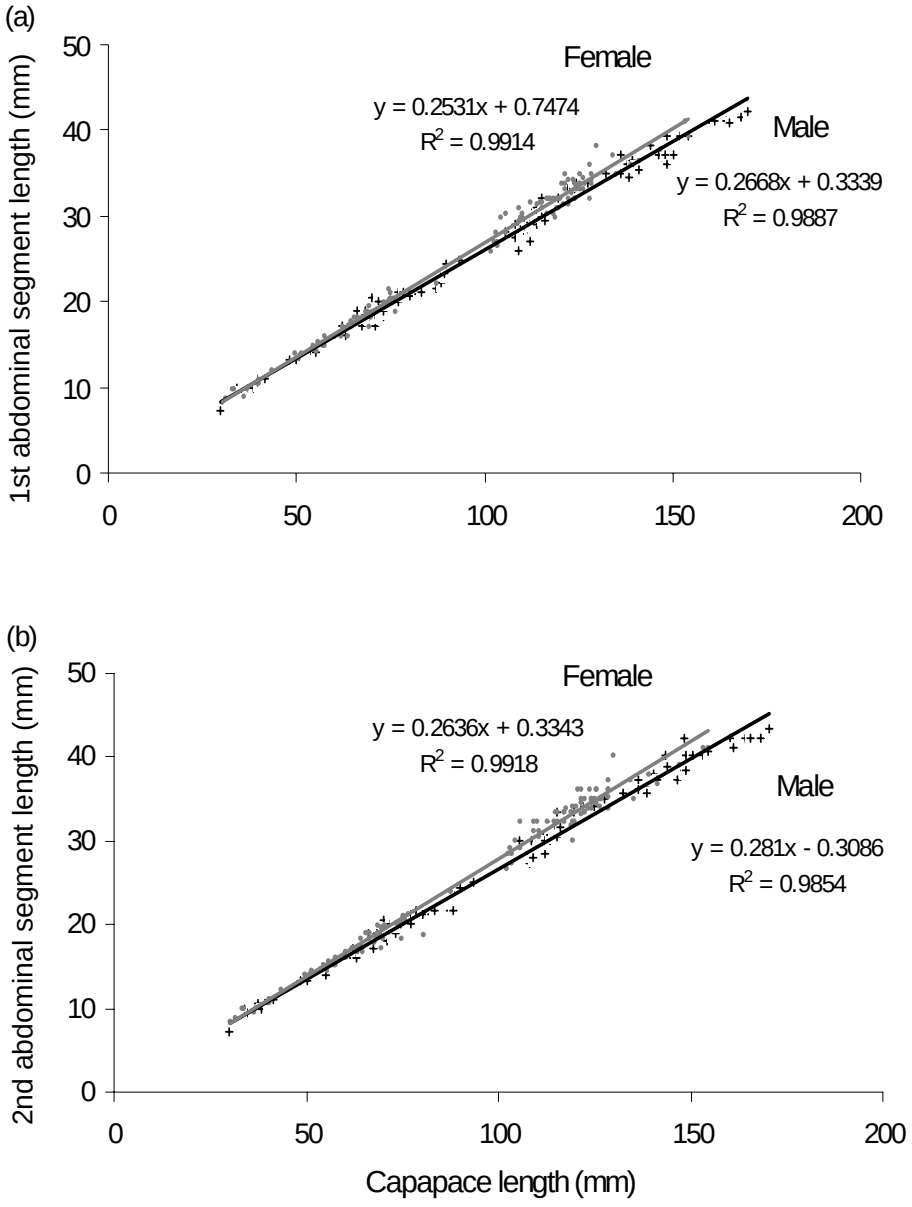

Fig. 3.4 The relationship between carapace length (mm) of male (n = 116) and female (n = 122) *J. edwardsii* and the length of the (a) 1st and (b) 2nd abdominal segment (mm).

The slopes of the regressions between CL and 1st abdominal segment width differed significantly between sexes ($F_{1,232}$ = 503.47, *P*<0.0001, Fig. 3.5a), and became discernibly wider in females in the 60 – 64.9 mm CL group and larger (ANOVA) i.e., midpoint 62.5 mm CL. Differences between the sexes were greater

for the width of the 2nd abdominal segment ($F_{1,232}$ = 624.74, P<0.0001, Fig. 3.5b) but the point of differentiation was the same at the 60 – 64.9 mm CL group (ANOVA) i.e., midpoint 62.5 mm CL.

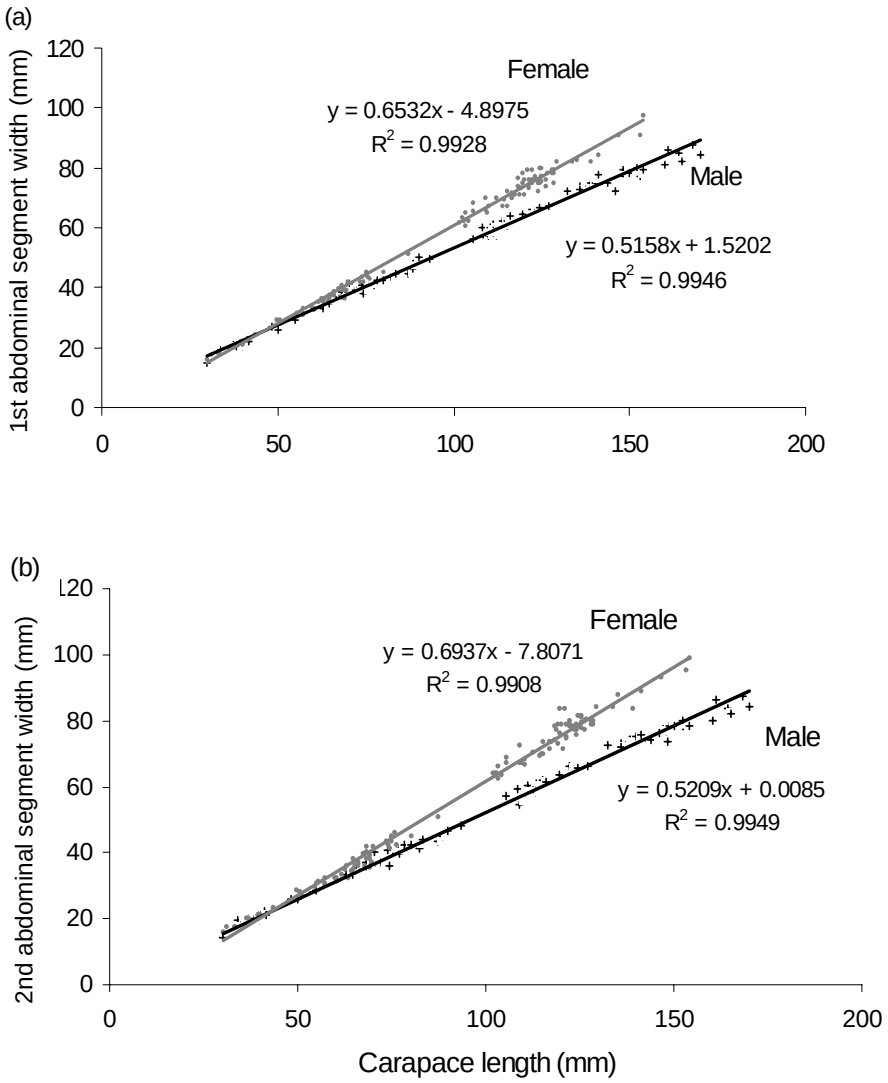

Fig. 3.5 The relationship between carapace length (mm) of male (n = 116) and female (n = 122) *J. edwardsii* and the width of the (a) 1st and (b) 2nd abdominal segments (mm).

There were strong linear relationships between CL and 2nd walking leg length, with different slopes evident between the sexes ($F_{1,232}$ = 311.63, P<0.0001, Fig. 3.6a). Males exhibited longer legs than females and the point of differentiation was

the 75 – 79.5 mm CL group (ANOVA) i.e., midpoint 77.5 mm CL. Differentiation between the sexes was improved using length measures of the 3rd walking leg ($F_{1,232}$ = 372.40, P<0.0001, Fig. 3.6b), but the point of differentiation was the same as the 75 – 79.5 mm CL group (ANOVA) i.e., midpoint 77.5 mm CL.

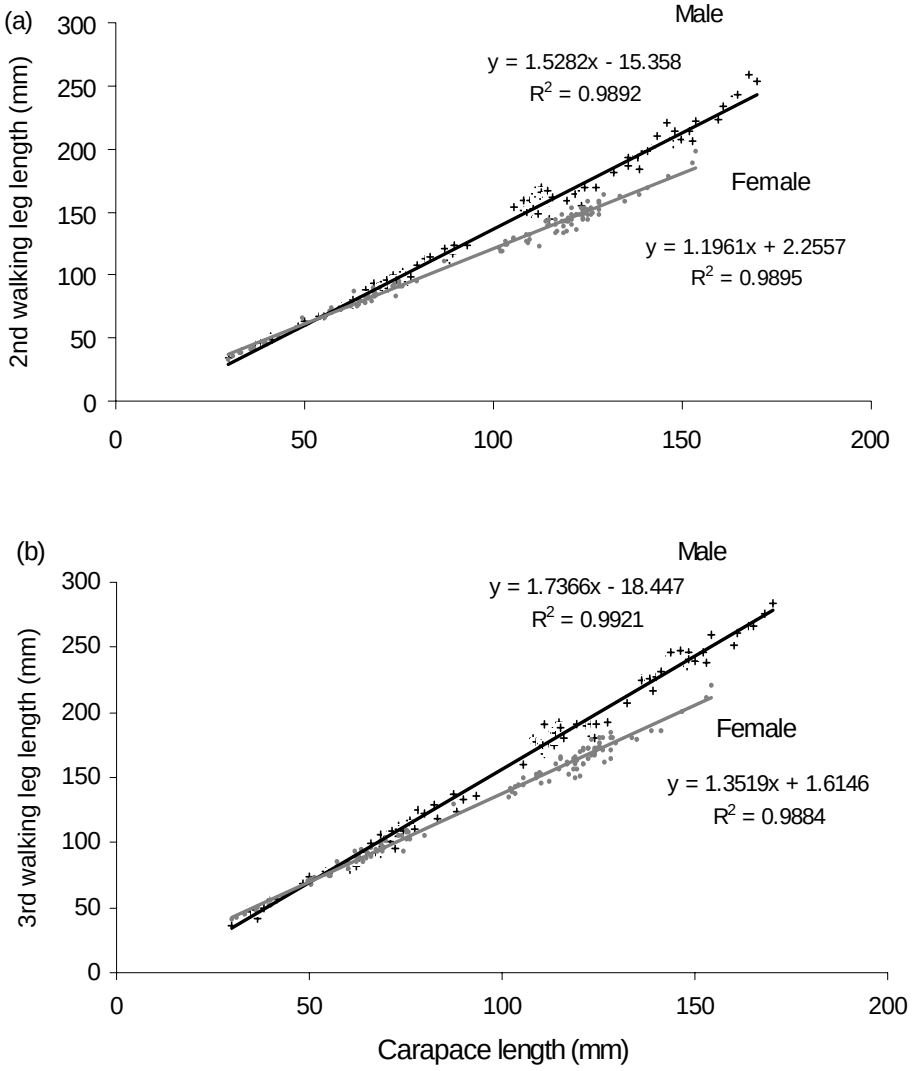

Fig. 3.6 The relationship between carapace length (mm) of male (n = 116) and female (n = 122) *J. edwardsii* and the length of the (a) 2nd and (b) 3rd walking legs (mm).

3.5.2. Hatch, viable fecundity and phyllosoma treatments

Hatch of phyllosoma from the 122 ovigerous females occurred from the 15th September to 29th November, 2001. A total of 30 females (chosen randomly from across the CL size range of the Bicheno group) were used to assess viable fecundity, with a reduced data set of 24 females used for phyllosoma treatments (egg diameter, phyllosoma size and LD-50).

The smallest ovigerous female had a CL of 58 mm, with the remaining 5 animals in this size class (55 – 59.9 mm) not ovigerous. The next size class (60-64.9 mm CL) contained 4 animals, all ovigerous, while subsequent 5 mm CL classes contained 90-100% ovigerous females. The SOM was therefore determined to be 62.5 mm CL. The size of animals sampled for viable fecundity varied from 58 – 152 mm CL, ww from 110 - 2025 g and viable phyllosoma numbers between 10 500 – 300 100. Viable fecundity was described by the equation $y = 0.1028*CL^{2.9204}$ ($R^2 = 0.9331$, $P< 0.0001$) (valid between 58 – 152 mm CL) (Fig. 3.7).

The range between minimum and maximum egg diameter, phyllosoma size and LD-50 was 0.70 – 0.83 mm, 1.88 – 2.08 mm and 8 – 19 d, respectively. There were significant correlations between viable fecundity and CL ($r > 0.9$) and phyllosoma size ($r > 0.7$) with reduced correlates for egg size ($r < 0.6$), stress indices ($-r < 0.6$) and survival in LD-50 ($r < 0.6$) (Table 3.2). There was a low but significant correlation between the results of the LD-50 and phyllosoma size ($r < 0.5$). Phyllosoma stress indices demonstrated a strong correlation with survival in the LD-50 ($r > -0.8$) and reduced correlates to egg size ($r < -0.5$) and phyllosoma size ($r < -0.6$). CL was correlated with egg size ($r < 0.6$) and phyllosoma size ($r > 0.7$).

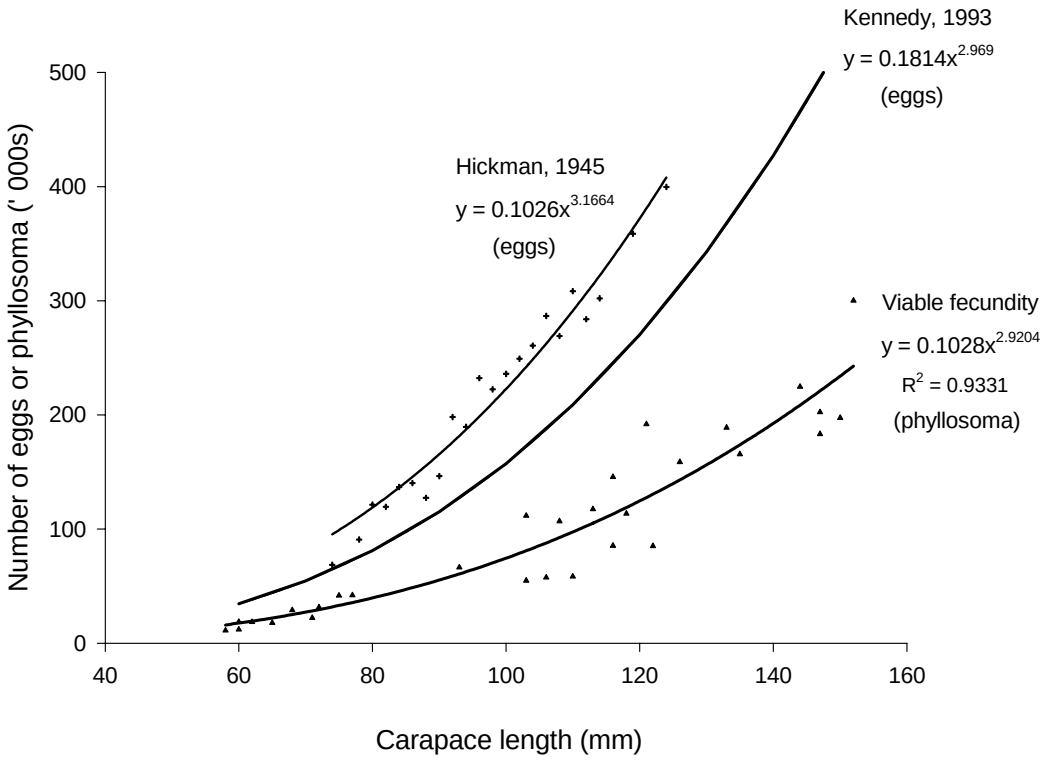

Fig. 3.7 The relationship between the carapace length (mm) of female *J. edwardsii* and the number of eggs (fecundity) as described by Hickman (1945), Kennedy (1993; unpublished Tasmanian fisheries data) or the number of phyllosoma (viable fecundity) noted in this study. Viable fecundity was from wild and captive ovigerous females from Bicheno held in captivity until hatch (n = 30).

Table 3.2 Significant correlates (r) of parameters associated with viable fecundity (the number of phyllosoma produced), egg and phyllosoma characteristics.

X value	Y value	r	Signif. Prob.
Viable fecundity	Carapace length	0.9202	0.0001
Viable fecundity	Egg size	0.5464	0.0070
Viable fecundity	Phyllosoma size	0.7370	0.0001
Viable fecundity	Stress index	-0.5612	0.0053
Viable fecundity	LD 50	0.5626	0.0124
LD 50	Phyllosoma size	0.4920	0.0171
Stress index	LD 50	-0.8936	0.0001
Stress index	Egg size	-0.4596	0.0274
Stress index	Phyllosoma size	-0.5312	0.0091
Carapace length	Egg size	0.5299	0.0093
Carapace length	Phyllosoma size	0.7034	0.0002

3.6. Discussion

The original study of *J. edwardsii* wild stocks in Tasmanian waters by Hickman (1945) found that the smallest ovigerous female had a SOM of 74 mm CL. Since then, SOM based on 50% maturity within a CL size class, is a regular feature of fisheries monitoring (Gardner et al., 2002). In Tasmanian waters, there is a positive relationship between SOM and temperature (Gardner et al., 2002), with larger SOM occurring in warm northern waters (Gardner, unpublished). In our study, the SOM for captive animals sourced from the Bicheno region as puerulus and held in captivity for 2 years was much lower (62.5 mm CL) than for animals from either their origin at Bicheno (93.2 mm CL) or Crayfish Point reserve (83.4 mm CL) the water source (ambient temperature) of the current study. This suggests that water temperature was not solely responsible for determining SOM, a finding also reported in other lobster fisheries (Kuhlmann and Walker II, 1999). Alternate explainations exist for smaller SOM in heavily exploited fisheries based on the removal of large mature individuals (Warner et al., 1977; Polovina, 1989), whereby early maturation as a result of heavy exploitation is a mechanism where smaller females are recruited to maintain egg production in the population (Polovina, 1989; Chubb, 1994; Pollock, 1997). A similar mechanism may be present in captive stock where large mature individuals are absent. It is interesting to note that across a range of environments and crustacean species there is little definitive knowledge of the cues that initiate the onset of maturity, a situation that needs to be remedied if the aquaculture potential of *J. edwardsii* is to be realized.

Synchronicity in the onset of SOM in captive stocks was high, the number of mature animals within a size cohort increased from 17% at 55-59.9 mm CL to 100% at 60-64.9 mm CL in consecutive size classes. The typical pattern for larger wild stocks is for 3-5 consecutive cohorts to contain increasing numbers of ovigerous females before 50% of a size class is mature (Annala et al., 1980; MacDiarmid, 1989; Gardner et al., 2002). However, this pattern is not exclusive, synchrony in the attainment of SOM (60 – 75 mm CL) has been noted previously in small animals (Annala et al., 1980) and may constitute a standard pattern for small maturing lobsters.

Morphological changes to males and females were associated with increased CL. In animals of similar total length, males have a larger CL, and considering the relationship between CL and weight, they will also be heavier than females. The greater length and width of the female abdominal segments is a trait associated with egg bearing and the formation of an egg chamber (Berry, 1971; Frusher, 1999). In this study, the change in tail shape appears to be closely related to the estimated SOM of 62.5 mm CL, and is confirmed by the divergence of male and female tail widths at this size. The point of dimorphism between the sexes may provide a useful method to determine the SOM for females. The use of morphometrics has been predominately confined to the differentiation of male sexual characteristics (Kubo, 1938; Juinio, 1987). In spiny lobsters, this has been associated with allometric elongation of the 3rd walking leg (Kubo, 1938; Gordon, 1960; Berry, 1971; George and Morgan 1979; Juinio, 1987; Plaut 1993) providing males with an increased capacity to participate in courtship and mating rituals (Kubo, 1938; MacDiarmid, 1989; Berry, 1971). This was also evident during our study where dimorphism between the sexes was evident at 77.5 mm CL, within the range previously reported for SOM in male *J. edwardsii* (65-85 mm CL) (Fielder, 1964; MacDiarmid, 1989).

The sequential relationship between large females producing large phyllosoma from large eggs (Annala, 1991) was confirmed, as was the potential for larger larvae to result in better survival (MacDiarmid and Kittaka, 2000; Smith et al., 2003b). The relationship between broodstock and larval size is an important finding, and provides an additional tool for selecting competent larvae for culture. During larval culture over successive stages, larger newly-hatched larvae become larger Stage II larvae, and so on. This is a typical growth pattern for crustaceans (Kunish and Anger, 1984; Lovrich and Vinuesa, 1995), where body size at ecdysis is proportional to the size of their previous exoskeleton. This is a factor especially important when selecting lobster larvae for culture, where the larval phase encompasses 11 moult stages, numerous instars and takes in excess of 300 days to complete (Kittaka, 1994).

Two measures of phyllosoma competency at hatch were used: an activity test and a LD–50. The activity test was virtually instantaneous (1 h) and proved more

effective in predicting larval performance during long-term culture than the LD–50. This is possibly due to an extended time-frame available during an LD-50 under which extraneous factors, such as bacterial contamination, may influence test results. The ability of larvae to withstand osmotic stress has been previously linked with an animals endogenous lipid (Dhert et al. 1992a,b; Rees et al. 1994; Abi-ayad et al. 1995; Kanazawa 1997; Cavalli et al. 1999) and ascorbic acid (Merchie et al 1993) profiles. The roles of lipids in maintaining cell membrane structure and function (Sargent 1995) and ascorbic acid in collagen production and osmotic regulation (Moreau et al. 1998) are suggested as reasons for a degree of protection against osmotic stress.

The slope of the viable fecundity curve for captive stocks had a similar slope to the most recent fecundity estimate (eggs) within Tasmanian waters, which had an equation $y = 0.1814*CL^{2.969}$ (Kennedy, 1993; unpublished Tasmanian fisheries data) but at 44 – 46% of the elevation and even less than an earlier estimate by Hickman (1945). This indicates that viable fecundity data is lower than documented historically for egg counts (Hickman, 1945; initially *Jasus lalandii*, later *J. novaehollandiae*, now *J. edwardsii*). It is unclear whether this difference between viable fecundity and fecundity is real or a discrepancy due to different sample size, geographic location or counting methodology over time. Parallels can be found in historic New Zealand fecundity records (Bradstock, 1950); also with lower values reported in recent times (MacDiarmid, 1989). During our study, we noted that phyllosoma number was considerably lower than egg number based on Tasmanian fishery data (Hickman, 1945; Kennedy, 1993; unpublished Tasmanian fisheries data) and in a captive scenario is contrary to the suggestion that minimal egg loss or viablility occurs during incubation (Kennedy, 1993; unpublished Tasmanian fisheries data). Possible explanations for low phyllosoma numbers include seasonal differences in egg number due to environmental or nutritional factors (Kontara et al., 1997; Sampedro et al., 1997) or egg loss during incubation.

Viable fecundity (i.e., the number of phyllosoma) is influenced by two major components: egg loss during incubation and the failure of newly-hatched larvae to progress beyond the naupliosoma stage. Many hatch events contain a small proportion of naupliosoma in which development may stall. Such developmental

problems may account for up to 10% of the total hatch and be more prominent on the first or last day of hatch (unpublished data). This is a relatively small component of predicted viable fecundity; therefore, any large discrepancy in viable fecundity must be assigned to egg loss/mortality. Up to 20% of eggs may be lost to mid-incubation in wild New Zealand *J. edwardsii* (Annala and Bycroft, 1987) and significant and sometimes total loss of eggs in captivity (MacDiarmid and Kittaka, 2000). Perkins (1971) suggested that the amount of loss may be related to incubation duration and reported 36% egg loss in *Homarus americanus* during 9 months incubation. The 4-5 month egg incubation period in *J. edwardsii* may lead to similarly high egg losses. Egg loss in crustaceans appears to be typical, rather than the exception (Tuck et al., 2000). The results of this preliminary study into the status of phyllosoma numbers at hatch are deemed sufficient to warrant a dedicated investigation into egg loss during incubation and the factors contributing ultimately to viable fecundity.

3.7. Conclusion

Factors inducing SOM are largely undetermined in spiny lobster species, although there are benefits for the wild fishery and aquaculture in large females producing large, viable phyllosoma. There were clear allometric relationships in both male and female *J. edwardsii* associated with SOM, which in the female provides potential correlates for use in assessing maturity in the absence of eggs or ovigerous setae. This study emphasized the importance of comparisons between fecundity as measured by egg production and viable phyllosoma at hatch. In this instance viable fecundity was below expected fecundity, whether due to altered wild estimates or primarily the result of natural or captivity-induced egg loss, and an issue worthy of dedicated comparative studies.

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4. Chapter Four - Phyllosoma Diet (a)

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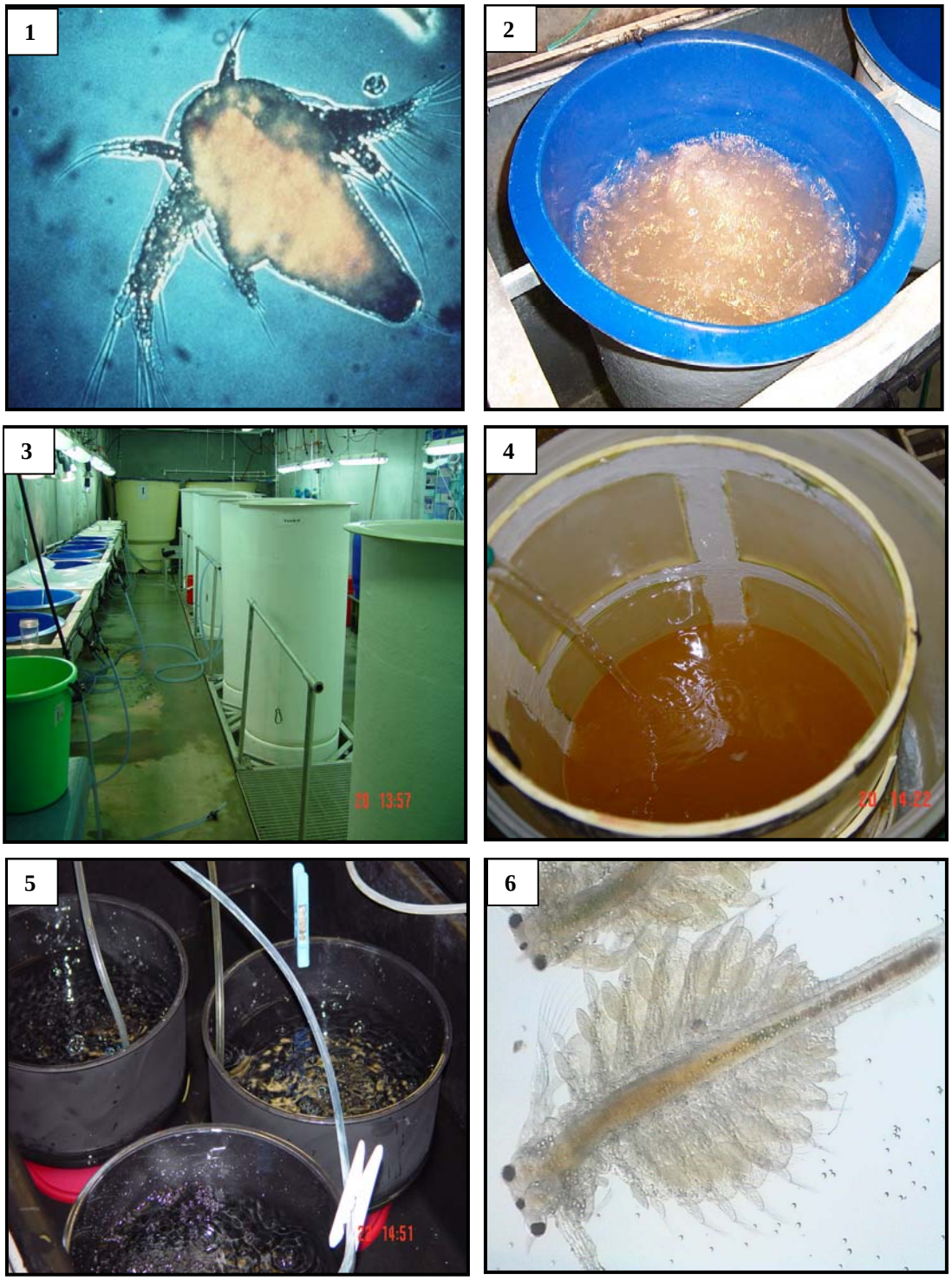

Plate 4. *Artemia* gut ingestion and evacuation study using plastic beads. (1) Instar I *Artemia* nauplii. (2) An *Artemia* hatching vessel. (3)The *Artemia* culture facility at the Tasmanian Aquaculture & Fisheries Institute, Marine Research Laboratories showing enrichment cones on the left-hand side and grow-out tanks in the centre. (4) *Artemia* biomass being harvested. (5) Experimental 3 L *Artemia* enrichment vessels. (6) Gut evacuation of juvenile *Artemia* using 20-40 μm plastic beads, note the beads in the gut cavity as well as in the water around the *Artemia.*

4.1. Abstract

Some larval crustaceans, including the spiny lobster *Jasus edwardsii*, tear *Artemia* to pieces before ingestion; this results in the loss of gut content, which may partly negate the benefits of enrichment with essential fatty acids (EFA). Therefore, the maintenance of gut content and how that affected the lipid composition of juvenile *Artemia* (5 day old) was examined by starvation alone or starvation with forced gut evacuation using 20-30 µm plastic beads. *Artemia* gut content at 3 h and 6 h after the completion of feeding did not contribute significantly to the total lipid or fatty acid profiles of the *Artemia*. *Artemia* subjected to starvation alone (without beads) failed to evacuate their gut over the 6 h starvation period suggesting they require the intake of suitable sized particulate matter to undertake gut evacuation. To assess the uptake of EFA in metanauplii (Day 2) and juveniles, an enrichment diet containing high levels of arachidonic [AA, 20:4n-6] and eicosapentaenoic acid [EPA, 20:5n-3] was compared to a basal oat-based diet and a commercial oil emulsion high in docosahexaenoic acid [DHA (22:6n-3]. Both AA and EPA were increased in juvenile *Artemia* within a 24 h enrichment period at a rate proportional to their inclusion in the enrichment, while DHA was incorporated to a lesser degree. For all three EFA, the percentage loss during 6 h starvation was small, but was greater for DHA than EPA or AA. Juvenile *Artemia*, a life stage seldom used in feeding regimes, have the ability to assume the AA and EPA profile of their dietary source. The ability to produce a larger food source with a non-traditional fatty acid profile may be valuable for a number of larval crustacean and fish species. As is the knowledge that minimum gut evacuation occurs in the absence of feeding stimuli, although inconsequential to the uptake of fatty acids in this instance prolonged gut resident time may be important to improving the assimilation of other nutritional factors.

Keywords: juvenile *Artemia*, gut evacuation, arachidonic acid, eicosapentaenoic acid, starvation, spiny lobster, phyllosoma.

4.2. Introduction

Many enrichment protocols for *Artemia* in the past have centered on newly-hatched nauplii and in particular, on how to achieve and maintain high levels of docosahexaenoic acid [DHA, 22:6n-3], eicosapentaenoic acid [EPA, 20:5n-3], and increase the DHA:EPA ratio (Watanabe et al., 1982; Rees et al., 1994; Rasowo et al., 1995; Narciso et al., 1999). While this is a suitable line of investigation for many cultured marine finfish and crustacean species, it may not be suited to phyllosoma larvae of the spiny lobster *Jasus edwardsii* (Phleger et al., 2001), a species under investigation for culture in Australia, New Zealand and Japan. In the wild, phyllosoma larvae of spiny lobster prey on a variety of invertebrates (Shojima, 1963; Thomas, 1963). However it has been shown, that they will reach metamorphosis to puerulus on a diet consisting solely of *Artemia* (Kittaka et al., 1988; Tong et al., 1997). Optimal growth and survival of phyllosoma are obtained when feeding juvenile *Artemia* (5 day old) of at least 1.5 mm in length (Tong et al., 1997; Ritar, 2001). It is also likely that they have a requirement for an enrichment product with a greater emphasis on arachidonic acid (AA, 20:4n-6) and in particular the EPA:AA ratio (Smith, 1999; Phleger et al., 2001). This is in contrast to current enrichment products that are targeted primarily towards the uptake of DHA and EPA in *Artemia* nauplii and metanauplii (2 day old) (Evjemo et al., 1997; McEvoy and Sargent, 1998; Narciso et al., 1999).

An understanding of the rate of incorporation and loss of essential fatty acids (EFA) in *Artemia* nauplii and metanauplii during enrichment and subsequent starvation has assisted in the development of feeding regimes targeted towards finfish (Estévez et al., 1998; Evjemo et al., 2001). While some research has been conducted on enrichment of juvenile *Artemia* (Dhont et al., 1991) there has been little emphasis on AA. The use of enriched juvenile *Artemia* will provide another food source available for crustacean and fish species (Naessens et al., 1997; Wouters et al., 1999; Smith, 1999) while enhancing existing knowledge based primarily on *Artemia* nauplii and metanauplii (Rasowo et al., 1995; Evjemo et al., 1997; Sorgeloos et al., 1998; Han et al., 2000; Evjemo et al., 2001).

A number of larval crustacean species (Abrunhosa et al., 1997; Crain, 1999; M. Nelson and S. Cox, pers com) tear large prey organisms into smaller pieces before consuming them, which may result in significant release and loss of material, particularly gut contents, into the surrounding aqueous environment. The benefits of short-term enrichment where a proportion of the enrichment may reside in the gut of large *Artemia* with little incorporation into cellular tissue could be negated with this type of feeding. Our observations (unpublished) suggest that juvenile *Artemia* starved for up to 24 h fail to fully evacuate their gut cavity. The food was observed as a distinctive coloration, but whether the gut content contributes significantly to the *Artemia* lipid and fatty acid content at this point is unknown.

The purpose of this study was to determine whether gut content after short-term enrichment was an important part of the overall composition of juvenile *Artemia*, and to approximate the fatty acid profile of newly hatched spiny lobster phyllosoma, in particular AA. A novel method is described whereby the food in the gut is evacuated and replaced by inert plastic beads. The target lipid enrichment profiles for *Artemia* were drawn from studies by Smith (1999) and Phleger et al. (2001) on the lipid class and fatty acid profiles of spiny lobster phyllosoma.

4.3. Materials and Methods

4.3.1. Artemia ongrowing

Decapsulated *Artemia* cysts (INVE, Great Salt Lake Prime Gold) were hatched at 28 ± 1°C in 50 L white fiberglass cones in 0.2 µm filtered brackish water (27 ± 1‰). At 24 h, newly hatched *Artemia* nauplii were removed from the hatching containers, rinsed in freshwater for 2 min and transferred to 1000 L conical tanks containing 200 L of filtered seawater (0.2 µm filtered, 34 ± 1‰, 28 ± 1°C). This volume was progressively increased to 1000 L over a 5-day ongrowing period providing a final *Artemia* density of 4 ml^{-1} with a total length of 1.5 ± 0.23mm (mean ± sem). During ongrowing for the gut evacuation trial, *Artemia* were fed with an “oat bran diet” consisting (w/w) of 75% oat bran, 12% wheat germ, 7%

lecithin and 6% fish oil (Fishaphos, Felton Grimwade and Bickford Pty Ltd, Victoria). The fish oil contained (v/v) 17% EPA and 11% DHA. *Artemia* used for the latter enrichment trial received only, oat bran: wheat germ: lecithin (OWL, 50:6:4) during the ongrowing period. *Artemia* diet was added to the water in the 1000 L tanks three times daily at a rate to maintain a Secchi depth of 25–30 cm.

4.3.2. Gut evacuation trial

Juvenile *Artemia* in 1000 L batch cultures were harvested after ongrowing for 5 days, they did not undergo a period of enrichment. An initial sample (0 h) of 32 000 *Artemia* was removed, rinsed and partitioned into 4 subsamples (fresh and lyophilized samples in duplicate). A further 80 000 *Artemia* were partitioned into either seawater alone without beads (w/o beads - starvation) or seawater with beads (w/beads – forced gut evacuation) at a density of 8 ml^{-1}. The experimental treatments were conducted in white plastic containers holding *Artemia* in 2.5 L of 0.2 μm filtered seawater (28°C and 34 ‰) in duplicate. Individual plastic beads (20–30 µm diameter; Ionics Inc, USA) were suspended in seawater at a density of 1.0×10^6 L^{-1} (beads weighed 4.74 ± 0.19 ng $bead^{-1}$). Counting of beads in the water was undertaken at 0 h, 3 h and 6 h.

4.3.3. 24 h Artemia enrichment experiment

Three enrichments were used to examine the uptake of essential fatty acids (EFA) in juvenile *Artemia*, and consisted (w/w) of:

- OWL - oat bran: wheat germ: lecithin (50:6:4)
- OWL+OIL - oat bran: wheat germ: lecithin: oil (50:6:4:40). The oil consisted (v/v) of a 4.5:1 ratio of Max EPA (containing 18% EPA and 12% DHA, Martek Biosgenes, USA) and ARASCO (containing 40% AA; Martek Biosgenes, USA).
- A1 DHA Selco (INVE Group, Belgium).

The ratio and level of oil inclusion in OWL+OIL were estimated from examination of oil incorporation in the gut evacuation study. The oat-based diets were prepared daily by blending ingredients suspended in 500 ml of seawater in a

household blender (high speed 15 min, Sunbeam, Australia) and large oat particles were removed by sieving (63 µm screen). A1 DHA Selco was prepared according to the manufacturer's directions. All enrichments were applied at a rate of 0.6g L^{-1}.

As for the gut evacuation trial, the 24 h enrichment experiment utilized *Artemia* ongrown in 1000 L batch culture systems. *Artemia* were harvested on Days 1 and 4 of ongrowing and partitioned into 1 L beakers at a density of 30 000 *Artemia* metanauplii per beaker and 10 000 *Artemia* juveniles per beaker, respectively. Twenty-seven beakers were utilized for each *Artemia* age class; these were divided equally into three groups and enriched with 1 of 3 diets for 24 h. After 24 h, triplicate samples for each age class and enrichment were rinsed and stored for analysis (9 beakers per age class). *Artemia* in the remaining beakers were rinsed and resuspended in either seawater alone without beads (w/o beads) or seawater with beads (w/beads) for a further 6 h. After 6 h, all treatments were terminated, rinsed and stored for analyses.

4.3.4. Lipid class and fatty acid analysis

In general, *Artemia* and feed samples were filtered through 4.7 cm Whatman glass filters (GF/F) and rinsed with 0.5 M ammonium formate. Filters were lyophilized overnight prior to analysis. Dry weight of the samples ranged from 5.6–262.5 mg. Additionally, the method of sample preparation (fresh or lyophilized) was examined to determine whether there was any loss or modification in fatty acid and lipid class composition. At 0 h in the 'gut evacuation trial', duplicate fresh samples were extracted immediately with solvent followed by analysis while another set of duplicate samples were frozen and lyophilized before extraction.

Samples were quantitatively extracted using a modified Bligh and Dyer (1959) one-phase methanol/chloroform/water extraction (2:1:0.8, by vol); each sample was extracted overnight and the phases were separated the following day by addition of chloroform and water (final solvent ratio, 1:1:0.9, v/v/v, methanol/chloroform/water). The total solvent extract was concentrated (i.e. solvents removed *in vacuo*) using rotary evaporation at 40°C and lipid content determined gravimetrically; weights of lipid were 0.8–49.7 mg. Lipid class

analyses were conducted within three days with samples stored in a known volume of chloroform.

An aliquot of the total solvent extract was analyzed using an Iatroscan MK V TH10 thin-layer chromatography–flame-ionization detector (TLC–FID) analyzer (Tokyo, Japan) to determine the abundance of individual lipid classes (Volkman and Nichols, 1991). Samples were applied in duplicate or triplicate to silica gel SIII Chromarods (5 µm particle size) using 1 µl disposable micropipettes. Chromarods were developed in a glass tank lined with pre-extracted filter paper. The solvent system used for the lipid separation was hexane/diethyl ether/acetic acid (60:17:0.2, by vol), a mobile phase resolving non-polar compounds such as wax ester (WE), triacylglycerol (TAG), free fatty acid (FFA) and sterol (ST). A second non-polar solvent system of hexane/diethyl ether (96:4 vol/vol) was also used for selected samples to separate hydrocarbon from WE. After development, the chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. The FID was calibrated for each compound class [phosphatidylcholine, cholesterol, cholesteryl ester, oleic acid, squalene, WE (derived from fish oil) and TAG (derived from fish oil); 0.1–10 µg range]. Peaks were quantified on an IBM compatible computer using DAPA software (Kalamunda, Western Australia). Iatroscan results are generally reproducible to ± 5% or better for individual classes (Bakes et al., 1995).

Fatty acid methyl esters (FAME) were extracted from an aliquot of the total solvent extract treated with methanol/hydrochloric acid/chloroform (10:1:1, by vol; 80°C, 2 h). FAME were extracted into hexane/chloroform (4:1, v/v, 3 x 1.5 ml) and then treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA, 50 µl, 60°C, 1 h) to convert sterols to their corresponding TMSi (trimethylsilyl) ethers.

Gas chromatographic (GC) analyses of FAME and sterols were performed with a Hewlett Packard 5890A GC (Avondale, PA) equipped with an HP-5 cross-linked methyl silicone fused silica capillary column (50 m × 0.32 mm i.d.), an FID, a split/splitless injector and an HP 7673A auto sampler. Hydrogen was the carrier gas. Following addition of methyl tricosanoate internal standard, samples were injected in splitless mode at an oven temperature of 50°C. After 1 min, the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1} and

finally to 300°C at 5°C min^{-1}. Peaks were quantified with Waters Millennium software (Milford, MA, USA). Individual components were identified using weight spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. GC results are subject to an error of ± 5% of individual component abundance.

GC–weight spectrometric (GC–MS) analyses were performed on a Thermoquest GCQ GC-weight spectrometer (Austin, TX, USA) fitted with an on-column injector. The GC was fitted with a capillary column similar to that described above.

4.4. Statistical analyses

Statistical analyses were conducted using one-way analysis of variance with Tukey-Kramer HSD tests used for post-hoc comparison (Sokal and Rohlf, 1995). Arcsin√ transforms were performed on percentage data. $P < 0.05$ were considered significantly different. Data are presented as mean ± *s.d.*, unless stated. Statistics were executed using JMP version 3.2.1. (SAS Institute Inc.).

4.5. Results

4.5.1. Gut evacuation of Artemia with inert plastic beads

Juvenile *Artemia* readily consume plastic beads giving the appearance of a gut cavity full of beads (Fig. 4.1). The mean number of beads counted in the gut cavity of a 1.5mm, 5-day old juvenile *Artemia* after 6 h exposure to beads was 67 ± 6. At 0 h, there was 1.0 x 10^6 beads L^{-1} present in the water column. After 3 h, the number of beads suspended in the water column was reduced to 0.533 x 10^6 ± 0.012 beads L^{-1}, which did not differ significantly from the count at 6 h (0.535 x 10^6 ± 0.028 beads L^{-1}). Uptake of beads into the *Artemia* was therefore approximately 50% of the available beads in the water column at 3 h and 6 h. More than 50% of the beads in the water column at 3 h were fouled with fecal material indicating the passage of beads through the *Artemia* took less than 3 h. The distinctive brown-yellow

coloration previously evident in the gut of all *Artemia* was reduced to negligible levels (visual observation) in the w/ beads treatment after 6 h, but not at 3 h. In *Artemia* in the w/o beads treatment, there was no visible reduction in the gut coloration at 3 h or 6 h compared to 0 h. Only a minor amount of fecal material was detected in the water column during the 6 h starvation period (*Artemia* w/o beads) indicating that only minimal voiding of the gut contents into the water column had occurred in this treatment.

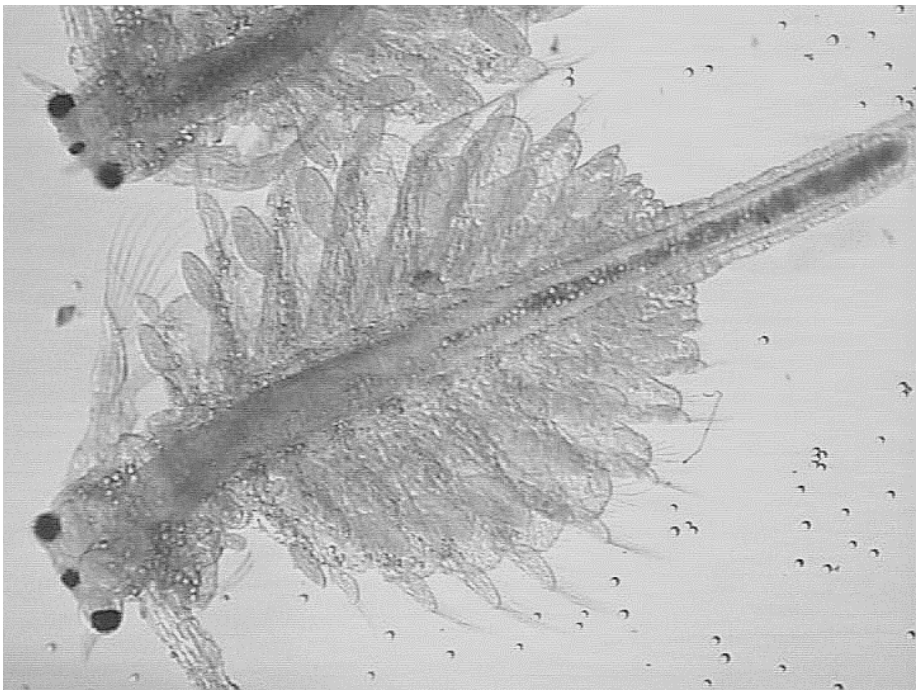

Fig. 4.1 Within a few minutes of suspending 20-30 um plastic beads in the water column, they are visible within the gut cavity of *Artemia* due to continuous mechanism of filtration and ingestion.

4.5.2. Juvenile Artemia - lipid class and fatty acid composition

The method of sample preparation (freshly prepared or lyophilized) did not significantly alter the lipid content nor the % of lipid class extracted (9.4 ± 2.1 mg g^{-1} wet weight (ww) compared to 7.7 ± 1.5 mg g^{-1} ww, respectively, Table 4.1). Due to the method of sample preparation where freshly prepared samples were not dried before extraction, only wet weight is presented in this section. Although a decrease in lipid content was observed in *Artemia* between 0 h, 3 h and 6 h, this was not significant. There was no difference in the lipid class composition in the treatments,

with or without beads at different sampling times. Polar lipid (PL) was the major lipid class (LC), comprising 53-58% of total lipid, followed by (TAG) with 39-44% of total lipid. All samples contained low levels of sterol (ST) (1.8-2.1%), and free fatty acid (FFA) (0.5-1.1%).

Table 4.1 Lipid class composition (percentage of total lipids), lipid content (wet weight) and percentage composition of major (>2%) and essential fatty acids in juvenile *Artemia* (1.5mm long). At time 0 h freshly prepared and lyophilized samples were analyzed. Lyophilized *Artemia* samples were used thereafter at 3 h and 6 h, during forced gut evacuation with beads (w/beads) or starvation without beads (w/o beads). Data are presented as mean ± *s.d.*; $n = 2$.

	0 h		3 h		6 h	
Lipid	Fresh	Lyophilized	w/ beads	w/o beads	w/ beads	w/o beads
Triacylglycerol	43.8 ± 0.4	44.4 ± 2.6	40.8 ± 1.5	40.9 ± 4.8	43.1 ± 2.1	39.0 ± 2.4
Free fatty acid	1.1 ± 0.2	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.9 ± 0.2	0.9 ± 0.2
Sterol	1.9 ± 0.2	1.9 ± 0.0	1.8 ± 0.7	2.1 ± 0.2	2.1 ± 0.7	1.9 ± 0.2
Polar lipid	53.1 ± 0.8	53.2 ± 2.8	56.7 ± 0.7	56.4 ± 4.4	53.9 ± 3.0	58.2 ± 2.4
Lipid mg g^{-1} (ww)	9.4 ± 2.1	7.7 ± 1.5	6.1 ± 1.1	6.1 ± 0.8	5.5 ± 0.1	4.2 ± 1.5
Fatty acid[1]						
16:0	12.3 ± 0.2*	11.8 ± 0.0	12.3 ± 0.1	12.2 ± 0.2	12.6 ± 1.0	12.2 ± 0.1
18:2n-6	32.3 ± 0.4*	33.8 ± 0.1	31.6 ± 1.6	32.4 ± 0.2	31.1 ± 0.5	32.5 ± 0.3
18:1n-9c/18:3n-3[2]	27.2 ± 0.5	28.0 ± 0.4	27.0 ± 1.0	27.7 ± 0.3	27.0 ± 0.6	28.0 ± 0.2
18:1n-7c	4.3 ± 0.2	4.4 ± 0.1	4.1 ± 0.3	4.3 ± 0.1	3.9 ± 0.5	4.0 ± 0.3
18:0	6.3 ± 0.1*	5.8 ± 0.1	6.4 ± 0.2	6.4 ± 0.1	7.1 ± 0.5	6.6 ± 0.1
20:4n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0
20:5n-3	3.0 ± 0.0	3.1 ± 0.1	3.0 ± 0.0	3.0 ± 0.1	3.1 ± 0.1	3.1 ± 0.2
22:6n-3	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0
Other	13.1	13.1	14.1	12.6	13.8	12.2
∑ SFA[3]	21.7 ± 1.0	20.7 ± 0.1	21.5 ± 1.2	21.3 ± 0.8	22.4 ± 1.4	21.1 ± 0.0
∑ MUFA	34.8 ± 0.5	35.5 ± 0.3	34.3 ± 1.4	35.0 ± 0.3	33.8 ± 1.2	34.9 ± 0.2
∑ PUFA	39.8 ± 0.5*	41.4 ± 0.3	40.4 ± 0.0	40.0 ± 0.7	39.6 ± 1.4	40.3 ± 0.4
∑ n-3	5.2 ± 0.3	4.9 ± 0.1	5.3 ± 0.2	4.9 ± 0.3	5.4 ± 0.5	5.4 ± 0.5
∑ n-6	34.2 ± 0.6*	35.9 ± 0.0	33.8 ± 1.4	34.4 ± 0.4	33.3 ± 1.0	34.2 ± 0.1
∑ n-3/n-6	0.2	0.1	0.2	0.1	0.2	0.2
EPA/AA	5.1	5.5	5.4	5.2	5.5	5.3
DHA/EPA	0.3	0.3	0.3	0.3	0.3	0.3

[1]Other fatty acids at <2%:14:0, i15:0, 16:1n-9c, 16:1n-7c, C_{16} PUFA, i17:0, 17:0, 18:3n-6, 18:4n-3, 20:1n-11c, 22:0, 22:5n-3 and C_{22} PUFA.
[2] Under GC these two components coeluted. GC-MS analysis showed that 18:1n-9c was the predominant component.
[3]SFA saturated fatty acid; MUFA monounsaturated fatty acid; PUFA polyunsaturated fatty acid.
* Signifies a significant difference between freshly prepared or lyophilized samples at time 0.

There were only minor significant differences between the fatty acid profiles of freshly extracted and lyophilized *Artemia*, and did not include the EFAs (Table 4.1). It was therefore considered that either method was appropriate for subsequent analyses, lyophilized samples were used thereafter. There were no significant differences in the fatty acid profiles of any of the lyophilized samples across all

sampling times either w/beads or w/o beads. The essential fatty acids, AA, EPA and DHA, were present as minor components (0.6-3.1%) of the total fatty acid profile.

4.5.3. Artemia size

There was a significant increase in *Artemia* length following 24 h enrichment for each age class, but there was no significant difference between enrichment treatments. *Artemia* total length at Day 1 and 4 was 0.72 ± 0.01 and 1.38 ± 0.01 mm, respectively, and following 24 h enrichment total length increased to 0.82 ± 0.02 and 1.49 ± 0.03 mm, respectively (n = 15 for all *Artemia* measures).

4.5.4. Dietary lipid and fatty acid composition

The major lipid classes in the enrichment diets were TAG and PL (33-88% and 11-65% of total lipid, respectively; Table 4.2). ST and FFA were approximately 1% or less, and wax ester (WE) was only detected in the A1 DHA Selco diet (0.2%). The OWL+OIL and A1 DHA Selco diets were high in lipid (288 and 806 mg g^{-1} dry weight (dw), respectively) dominated by TAG (85 and 88%, respectively), in contrast to the PL-dominated (65%) OWL enrichment containing 165 mg g^{-1} dw of lipid. The dominant fatty acids in the OWL enrichment diet (Table 4.3), were 18:1n-9, 18:2n-6 and 16:0, while AA and EPA made substantial contributions to the OWL+OIL diet (8.9% and 11.7% respectively, Table 4.4), as did DHA to the A1 DHA Selco diet (20.7%, Table 4.5). The latter two diets contained substantially lower levels of 18:2n-6 and 18:1n-9 compared to that found in the OWL diet. The n-3/n-6 ratio of the 3 enrichment diets differed substantially. The OWL diet was dominated by high levels of n-6 fatty acids (n-3/n-6 ratio of < 0.1), whereas equal amounts of n-3 and n-6 fatty acids were present in the OWL+ OIL diet (1.1), and the n-3 class dominated the A1 DHA Selco diet (n-3/n-6 ratio of 3.1). The ratios of the n-3/n-6 fatty acids in the different diets were mirrored by their respective EPA:AA ratios.

Table 4.2 Lipid class composition (percentage of total lipids) and lipid content (mg g^{-1}) of enrichments and *Artemia* at Day 1 and Day 4, after 24 h enrichment (Day 2 and Day 5, respectively), and further 6h starvation. Newly-hatched spiny lobster phyllosoma profiles (target predator species) are included for comparison (Smith, 1999). Data are presented as mean ± *s.d.* (*n* = 3, except for starved 6 h, n = 6).

	Wax ester	Triacylglycerol	Free fatty acid	Sterol	Polar lipid	Lipid as mg g^{-1}
Phyllosoma	3.3	2.5	1.3	8.7	81.8	104.6
Enrichments[2]						
OWL	0.0 ± 0.0	33.4 ± 2.3	1.2 ± 0.3	0.7 ± 0.1	64.7 ± 2.5	164.5 ± 1.0
OWL+OIL	0.0 ± 0.0	87.8 ± 1.4	0.0 ± 0.0	1.1 ± 0.2	11.2 ± 1.6	287.8 ± 6.7
A1 DHA Selco	0.2 ± 0.0	85.3 ± 0.2	0.5 ± 0.0	0.5 ± 0.0	13.6 ± 0.1	806.1 ± 38.8
Day 1 *Artemia*	0.6 ± 0.2	[a] 45.0 ± 1.4	0.8 ± 0.2	[a] 2.3 ± 0.4	[a] 51.3 ± 1.9	[a] 149.6 ± 5.5
Day 2 *Artemia*						
24h OWL	0.4 ± 0.1 [a]	[a] 50.8 ± 3.6 [a]	1.7 ± 0.7	[ab] 2.8 ± 0.4	[b] 44.3 ± 3.4 [a]	[a] 202.9 ± 37.4
starved 6h	0.0 ± 0.0 [b]	57.4 ± 2.6 [b]	4.1 ± 2.0	2.8 ± 0.6	35.8 ± 2.8 [b]	202.2 ± 24.1
Day 2 *Artemia*						
24h	0.5 ± 0.1[a]	[b] 62.1 ± 3.4	3.8 ± 3.2	[a] 2.2 ± 0.1	[c] 31.5 ± 0.3	[a] 203.6 ± 15.1
starved 6h	0.2 ± 0.1[b]	66.6 ± 3.8	2.1 ± 1.5	2.1 ± 0.4	28.9 ± 3.2	264.3 ± 91.8
Day 2 *Artemia*						
24h DHA	0.7 ± 0.1 [a]	[b] 65.3 ± 1.5	5.0 ± 0.3 [a]	[b] 3.1 ± 0.2 [a]	[c] 26.0 ± 1.5	[b] 264.7 ± 17.3
starved 6h	0.2 ± 0.2 [b]	73.2 ± 9.4	1.5 ± 0.4 [b]	1.6 ± 0.6 [b]	23.4 ± 8.4	255.2 ± 28.8
Day 4 *Artemia*	0.3 ± 0.2	[a] 40.7 ± 1.7	[a] 1.2 ± 0.5	[a] 3.4 ± 0.3	[a] 54.5 ± 2.2	[a] 182.1 ± 34.3
Day 5 *Artemia*						
24h OWL	0.2 ± 0.2	[b] 68.4 ± 13.6	[a] 1.6 ± 1.0	[a] 1.9 ± 0.9	[b] 27.9 ± 11.7	[a] 186.4 ± 7.3
starved 6h	0.1 ± 0.1	46.6 ± 19.3	2.8 ± 0.9	3.0 ± 0.6	47.5 ± 18.7	151.0 ± 20.1
Day 5 *Artemia*						
24h	0.1 ± 0.2	[b] 70.6 ± 1.9	[ab] 1.8 ± 0.7	[ab] 2.2 ± 0.4	[b] 25.3 ± 1.8	[b] 258.9 ± 5.8
starved 6h	0.1 ± 0.1	69.2 ± 2.6	3.4 ± 2.3	1.8 ± 0.2	25.6 ± 0.8	242.6 ± 74.7
Day 5 *Artemia*						
24h DHA	0.0 ± 0.0	[b] 71.8 ± 3.3	[b] 4.4 ± 1.5 [a]	[b] 1.6 ± 0.5	[b] 22.3 ± 1.3	[b] 296.5 ± 31.7
starved 6h	0.0 ± 0.0	74.8 ± 2.7	1.9 ± 0.7 [b]	1.2 ± 0.1	22.1 ± 2.7	283.8 ± 34.8

[1]In phyllosoma samples, wax ester also contains hydrocarbon; sterol contains diacylglycerol.
[2]Enrichment composition: OWL - oat bran: wheat germ: lecithin (50:6:4); OWL+OIL - oat bran: wheat germ: lecithin: oil (50:6:4:40). Oil composed of Max EPA: ARASCO - 4.5:1, A1 DHA Selco (INVE Group, Belgium).
[3]dw = dry weight.
Different superscript preceding data values denote significant differences between Day 1 or Day 4 ongrown *Artemia* and *Artemia* after 24h enrichment.
Different superscripts following data values denote significant differences between 24 h enrichment and 6 h starvation within a dietary group.

Table 4.3 Percentage composition of major (>2%) and essential fatty acids in OWL enrichment, *Artemia* at Day 1 and Day 4, after 24 h enrichment with OWL (Day 2 and Day 5, respectively) and 6h starvation. Newly-hatched spiny lobster phyllosoma profiles (target predator) are included for comparison (Smith 1999). Data are presented as mean ± *s.d.* (*n* = 3, except for starved 6 h, where n = 6). Enrichment composition: OWL - oat bran: wheat germ: lecithin (50:6:4).

Fatty acids (%)	Phyllosoma	OWL	Ongrown	24h enrichment	6h starved	Ongrown	24h enrichment	6 h starved
14:0	0.9	0.1 ± 0.0	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.2	1.8 ± 0.0 ^{a}	1.0 ± 0.1 ^{b}	1.2 ± 0.2 ^{b}
16:1n-7c	2.3	0.1 ± 0.0	3.2 ± 0.2 ^{a}	2.2 ± 0.0 ^{b}	2.3 ± 0.2 ^{b}	2.8 ± 0.0 ^{a}	1.5 ± 0.1 ^{c}	2.0 ± 0.1 ^{b}
16:0	13.1	19.1 ± 0.1	11.6 ± 0.1 ^{b}	11.0 ± 0.4 ^{b}	12.3 ± 0.5 ^{a}	12.3 ± 0.3 ^{a}	11.5 ± 0.2 ^{b}	12.5 ± 0.2 ^{a}
18:4n-3	0.0	0.0 ± 0.0	3.7 ± 0.1 ^{a}	1.7 ± 0.1 ^{b}	1.5 ± 0.2 ^{b}	3.5 ± 0.2 ^{a}	1.3 ± 0.0 ^{c}	1.7 ± 0.1 ^{b}
18:2n-6	1.8	44.4 ± 0.5	10.3 ± 0.3 ^{b}	25.2 ± 0.4 ^{a}	25.0 ± 1.3 ^{a}	22.8 ± 0.1 ^{c}	35.2 ± 0.7 ^{a}	30.6 ± 0.8 ^{b}
18:1n-9c/18:3n-3 [1]	13.2	30.1 ± 0.3	42.9 ± 0.6 ^{a}	36.5 ± 1.1 ^{b}	35.6 ± 1.3 ^{b}	29.5 ± 0.4 ^{b}	31.2 ± 0.8 ^{ab}	29.8 ± 1.2 ^{b}
18:1n-7c	4.8	1.2 ± 0.0	8.4 ± 0.2 ^{a}	6.3 ± 0.2 ^{b}	5.7 ± 0.4 ^{c}	5.9 ± 0.1 ^{a}	3.7 ± 0.1 ^{b}	4.3 ± 0.2 ^{b}
18:0	7.2	2.6 ± 0.0	5.8 ± 0.1 ^{b}	6.1 ± 0.4 ^{a}	6.2 ± 0.2 ^{a}	6.9 ± 0.0 ^{a}	5.5 ± 0.0 ^{b}	6.6 ± 0.2 ^{a}
20:4n-6	8.2	0.1 ± 0.0	0.6 ± 0.0 ^{b}	0.6 ± 0.1 ^{ab}	1.1 ± 0.8 ^{a}	1.2 ± 0.0 ^{a}	0.7 ± 0.0 ^{b}	1.2 ± 0.2 ^{b}
20:5n-3	20.3	0.1 ± 0.0	3.1 ± 0.0 ^{a}	2.0 ± 0.0 ^{b}	2.5 ± 0.9 ^{b}	2.8 ± 0.0 ^{a}	1.6 ± 0.1 ^{b}	2.5 ± 0.3 ^{a}
22:6n-3	11.3	0.0 ± 0.0	0.0 ± 0.0 ^{b}	0.3 ± 0.2 ^{a}	0.6 ± 0.5 ^{a}	1.2 ± 0.0 ^{a}	0.7 ± 0.1 ^{b}	1.1 ± 0.2 ^{ab}
Other[2]	16.9	2.2	9.6	7.3	6.4	9.3	6.1	6.5
∑ n-3	33.1	0.1 ± 0.0	7.5 ± 0.1 ^{a}	4.3 ± 0.2 ^{b}	4.8 ± 1.6 ^{b}	8.0 ± 0.3 ^{a}	3.8 ± 0.1 ^{c}	5.5 ± 0.6 ^{b}
∑ n-6	12.2	44.6 ± 0.4	11.5 ± 0.2 ^{b}	26.3 ± 0.6 ^{a}	26.6 ± 0.9 ^{a}	25.2 ± 0.1 ^{c}	36.6 ± 0.8 ^{a}	32.7 ± 0.6 ^{b}
∑ n-3/n-6	2.7	<0.1	0.7 ^{a}	0.2 ^{b}	0.2 ^{b}	0.3 ^{a}	0.1 ^{c}	0.2 ^{b}
EPA/AA	2.5	0.7	5.0 ^{a}	3.6 ^{b}	2.9 ^{b}	2.3 ^{a}	2.2 ^{b}	2.0 ^{b}
DHA/EPA	0.6	0.5	0.0 ^{b}	0.1 ^{a}	0.2 ^{a}	0.4	0.5	0.4
Total fatty acids (mg/g)			144.3 ± 10.6	167.5 ± 7.0	150.8 ± 19.4	138.4 ± 7.4	165.0 ± 14.0	139.5 ± 21.3

Different superscript denotes a significant difference between Day 1 or Day 5 *Artemia*, 24h enrichment and 6h starvation.
[1]Under GC, these two components coeluted. GC-MS analysis showed that 18:1n-9c was the predominant component.
[2]Other fatty acids include: i15:0, 16:1n-9c, C_{16} PUFA, i17:0, 17:0, 18:3n-6, 20:1n-11c, 22:0, 22:5n-3 and C_{22} PUFA.

Table 4.4 Percentage composition of major (>2%) and essential fatty acids in OWL+OIL enrichment, *Artemia* at Day 1 and Day 4, after 24 h enrichment with OWL+ OIL (Day 2 and Day 5, respectively) and 6h starvation. Newly-hatched spiny lobster phyllosoma profiles (target predator species) are included for comparison (Smith 1999). Data are presented as mean ± *s.d.* (*n* = 3, except for starved 6 h, where n = 6). Enrichment composition: OWL+OIL - oat bran: wheat germ: lecithin: oil (50:6:4:40). Oil composed of Max EPA: ARASCO - 4.5:1.

Fatty acid (%)	Phyllosoma	OWL+OIL	Ongrown	24h enrichment	6h starved	Ongrown	24h enrichment	6 h starved
14:0	0.9	5.6 ± 0.2	0.8 ± 0.0 [b]	2.1 ± 0.0 [a]	2.0 ± 0.5 [a]	1.8 ± 0.0 [b]	2.2 ± 0.0 [a]	2.3 ± 0.2 [a]
16:1n-7c	2.3	6.6 ± 0.6	3.2 ± 0.2 [b]	4.3 ± 0.3 [a]	4.6 ± 0.5 [a]	2.8 ± 0.0 [b]	4.8 ± 0.1 [a]	4.8 ± 0.2 [a]
16:0	13.1	17.1 ± 0.4	11.6 ± 0.1	11.7 ± 0.4	11.5 ± 1.6	12.3 ± 0.3 [a]	10.3 ± 0.1 [b]	11.9 ± 0.4 [a]
18:4n-3	0.0	0.0 ± 0.0	3.7 ± 0.1 [a]	1.9 ± 0.1 [b]	2.1 ± 0.2 [b]	3.5 ± 0.2 [a]	2.1 ± 0.1 [b]	2.0 ± 0.2 [b]
18:2n-6	1.8	10.0 ± 0.3	10.3 ± 0.3	10.3 ± 0.6	11.8 ± 1.1	22.8 ± 0.1 [a]	16.7 ± 0.2 [b]	16.9 ± 1.1 [b]
18:1n-9c/18:3n-3[1]	13.2	14.2 ± 0.2	42.9 ± 0.6 [a]	26.8 ± 0.3 [b]	28.3 ± 1.0 [b]	29.5 ± 0.4 [a]	22.2 ± 0.3 [b]	21.7 ± 4.1 [b]
18:1n-7c	4.8	2.8 ± 0.0	8.4 ± 0.2 [a]	6.0 ± 0.2 [b]	6.3 ± 0.2 [b]	5.9 ± 0.1 [a]	4.1 ± 0.1 [c]	5.0 ± 0.3 [b]
18:0	7.2	4.4 ± 0.1	5.8 ± 0.1 [a]	5.3 ± 0.3 [b]	5.3 ± 0.4 [ab]	6.9 ± 0.0 [a]	4.1 ± 0.0 [c]	5.3 ± 0.3 [b]
20:4n-6	8.2	8.9 ± 0.0	0.6 ± 0.0 [b]	7.4 ± 0.6 [a]	6.0 ± 2.5 [a]	1.2 ± 0.0 [c]	8.9 ± 0.2 [a]	7.5 ± 0.9 [b]
20:5n-3	20.3	11.7 ± 0.3	3.1 ± 0.0 [b]	9.9 ± 0.3 [a]	8.1 ± 3.2 [a]	2.8 ± 0.0 [c]	11.3 ± 0.1 [a]	9.5 ± 0.8 [b]
22:6n-3	11.3	7.5 ± 0.0	0.0 ± 0.0 [b]	5.2 ± 0.2 [a]	4.5 ± 1.1 [a]	1.2 ± 0.0 [c]	5.3 ± 0.4 [a]	4.3 ± 0.6 [b]
Other[2]	16.9	11.2	9.6	9.1	9.5	9.3	8.0	8.8
∑ n-3	33.1	23.0 ± 0.4	7.5 ± 0.1 [b]	18.7 ± 0.4 [a]	16.3 ± 2.2 [a]	8.0 ± 0.3 [c]	20.3 ± 0.5 [a]	17.2 ± 1.7 [b]
∑ n-6	12.2	20.5 ± 0.5	11.5 ± 0.2 [b]	19.0 ± 0.2 [a]	19.2 ± 2.2 [a]	25.2 ± 0.1	27.2 ± 0.2	25.9 ± 1.6
∑ n-3/n-6	2.7	1.1	0.7 [b]	1.0 [a]	0.8 [b]	0.3 [c]	0.7 [a]	0.7 [b]
EPA/AA	2.5	1.3	5.0 [a]	1.3 [b]	1.4 [b]	2.3 [a]	1.3 [b]	1.3 [b]
DHA/EPA	0.6	0.6	0.0 [b]	0.5 [a]	1.1 [a]	0.4	0.5	0.4
Total fatty acids (mg/g)			144.3 ± 10.6[a]	197.7 ± 10.2[b]	214.5 ± 12.2 [b]	138.4 ± 7.4 [a]	231.8 ± 26.0 [b]	139.5 ± 21.3 [a]

See Table 4.3 for additional information.

Table 4.5 Percentage composition of major (>2%) and essential fatty acids in A1 DHA Selco enrichment, *Artemia* at Day 1 and Day 4, after 24 h enrichment with A1 DHA Selco (Day 2 and Day 5, respectively) and 6h starvation. Newly- hatched spiny lobster phyllosoma profiles (target predator) are included for comparison (Smith 1999). Data are presented as mean ± *s.d.* (*n* = 3, except for starved 6 h, where n = 6). Enrichment composition: A1 DHA Selco (INVE Group, Belgium).

Fatty acid (%)	Phyllosoma	A1 DHA	Ongrown	24h enrichment	6h starved	Ongrown	24h enrichment	6 h starved
14:0	0.9	3.3 ± 0.0	0.8 ± 0.0 [c]	1.5 ± 0.0 [a]	1.2 ± 0.1 [b]	1.8 ± 0.0	1.6 ± 0.1	1.5 ± 0.1
16:1n-7c	2.3	4.2 ± 0.2	3.2 ± 0.2 [b]	3.6 ± 0.1 [b]	4.0 ± 0.1 [a]	2.8 ± 0.0 [c]	4.1 ± 0.1 [b]	4.4 ± 0.2 [a]
16:0	13.1	21.4 ± 0.6	11.6 ± 0.1 [c]	12.7 ± 0.4 [a]	10.7 ± 0.4 [b]	12.3 ± 0.3 [a]	11.0 ± 0.2 [b]	10.6 ± 0.3 [b]
18:4n-3	0.0	0.0 ± 0.0	3.7 ± 0.1 [a]	1.3 ± 0.0 [c]	1.4 ± 0.0 [b]	3.5 ± 0.2 [a]	1.6 ± 0.1 [b]	1.6 ± 0.1 [b]
18:2n-6	1.8	5.4 ± 0.2	10.3 ± 0.3 [ab]	8.9 ± 0.3 [b]	10.2 ± 0.6 [a]	22.8 ± 0.1 [a]	13.2 ± 0.1 [b]	14.2 ± 0.6 [b]
18:1n-9c/18:3n-3[1]	13.2	18.0 ± 3.0	42.9 ± 0.6 [a]	27.9 ± 0.1 [c]	30.6 ± 1.0 [b]	29.5 ± 0.4 [a]	24.0 ± 0.1 [b]	24.9 ± 0.8 [b]
18:1n-7c	4.8	3.0 ± 0.2	8.4 ± 0.2 [a]	5.6 ± 0.1 [c]	6.2 ± 0.3 [b]	5.9 ± 0.1 [a]	4.7 ± 0.2 [c]	5.0 ± 0.1 [b]
18:0	7.2	6.0 ± 0.2	5.8 ± 0.1 [a]	4.7 ± 0.2 [a]	4.3 ± 0.1 [b]	6.9 ± 0.0 [a]	4.2 ± 0.1 [b]	4.1 ± 0.2 [b]
20:4n-6	8.2	1.7 ± 0.1	0.6 ± 0.0	1.9 ± 0.0	2.1 ± 0.2	1.2 ± 0.0 [b]	2.7 ± 0.7 [a]	2.4 ± 0.2 [a]
20:5n-3	20.3	5.3 ± 0.3	3.1 ± 0.0 [b]	7.1 ± 0.1 [a]	7.7 ± 0.2 [a]	2.8 ± 0.0 [b]	8.7 ± 0.3 [a]	8.2 ± 0.3 [a]
22:6n-3	11.3	20.7 ± 0.6	0.0 ± 0.0 [c]	15.8 ± 0.3 [a]	12.4 ± 0.4 [b]	1.2 ± 0.0 [c]	15.4 ± 1.1 [a]	13.6 ± 0.7 [b]
Other[2]	16.9	11.0	9.6	9.0	9.2	9.3	8.8	9.5
∑ n-3	33.1	28.1 ± 1.1	7.5 ± 0.1 [b]	25.6 ± 0.4 [a]	22.8 ± 0.7 [a]	8.0 ± 0.3 [c]	27.0 ± 0.8 [a]	24.6 ± 0.8 [b]
∑ n-6	12.2	9.2 ± 0.4	11.5 ± 0.2 [a]	12.4 ± 0.3 [b]	13.9 ± 0.4 [b]	25.2 ± 0.1 [a]	17.8 ± 0.7 [b]	18.4 ± 0.8 [b]
∑ n-3/n-6	2.7	3.1	0.7 [a]	2.1 [b]	1.6 [b]	0.3 [c]	1.5 [a]	1.3 [b]
EPA/AA	2.5	3.2	5.0 [a]	3.7 [b]	3.6 [b]	2.3 [b]	3.2 [a]	3.4 [a]
DHA/EPA	0.6	3.9	0.0 [a]	2.2 [b]	1.6 [b]	0.4 [b]	1.8 [a]	1.6 [a]
Total fatty acids (mg/g)			144.3 ± 10.6 [a]	247.0 ± 31.0 [b]	245.1 ± 18.9 [b]	138.4 ± 7.4 [a]	261.0 ± 17.7 [b]	236.2 ± 54.1 [b]

See Table 4.3 for additional information.

4.5.5. Enriched Artemia - lipid and fatty acid composition

There were no significant differences across all enrichment treatments and *Artemia* age in the lipid class profiles for animals starved w/beads or w/o beads, so data from both starved treatments was pooled (n = 6) for comparisons with 24 h enrichment (Table 4.2). Lipid data for Stage I spiny lobster phyllosoma (obtained from wild caught ovigerous females), the target predator species are also included for comparison. As was seen in the enrichment profiles, TAG and PL in 24 h enriched *Artemia* (Day 2 and 5) were the dominant lipid classes. There were significant increases in the relative level of TAG in *Artemia* exposed to all enrichments after 24 h. The greatest increases were present in *Artemia* fed the OWL+OIL (38 and 73% Day 2 and 5, respectively) and A1 DHA Selco (41 and 76% Day 2 and 5, respectively) diets. As a result of the increased TAG, the percentage contribution of PL was significantly reduced in all 24 h enriched *Artemia*. All Day 5 *Artemia* contained between 7-18% more TAG than Day 2 *Artemia*.

After 24 h enrichment and with 6 h starvation, Day 2 OWL enriched *Artemia* demonstrated a further significant increase in TAG while PL decreased. No such changes were evident in TAG or PL in any of the other treatments at Day 2 or Day 5. A number of small but significant shifts in the minor lipid components (WE, FFA and ST) occurred in all three enrichment treatments after 6 h starvation. However, this was confined to Day 2 *Artemia*, and the FFA levels of A1 DHA Selco enriched Day 5 *Artemia.*

In Day 2 *Artemia*, the amount of lipid accumulated expressed as mg g^{-1} dw was significantly greater in animals enriched for 24 h with A1 DHA Selco, while in Day 5 *Artemia* there were significant increases in lipid dry weight in both OWL+OIL and A1 DHA Selco enriched animals. There was no significant change in lipid dry weight across any treatment or *Artemia* age with starvation.

The major fatty acids in Day 1 and Day 4 *Artemia* were 18:1n-9, 18:2n-6, 16:0, 18:1n-7, 18:0 (Tables 4.3-4.5). A number of these fatty acids were also prominent in the diets and subsequently in the 24 h enriched Day 2 and Day 5 *Artemia*. In particular, 18:2n-6 made a large contribution to the total fatty acid profile of the

OWL diet (44.4%) with subsequent transfer to both Day 2 and 5 *Artemia*. The OWL+OIL and A1 DHA Selco diets contained lower levels of 18:2n-6 (10.0 and 5.4%, respectively). However, in both cases 18:2n-6 still assumed major prominence in 24 h enriched *Artemia* (range of 8.9-10.3% in Day 2 *Artemia* and 13.2-16.9% in Day 5 *Artemia*).

OWL+OIL and A1 DHA Selco enriched *Artemia* incorporated increased proportions of EFA largely reflecting the profiles of their diets. There was a greater uptake of AA and EPA seen in Day 5 *Artemia* compared to Day 2 *Artemia,* while the uptake of DHA was similar for both Day 2 and 5 *Artemia*. Where these three EFA were present as minor enrichment components, such as in the OWL diet (0.1%), enrichment resulted in EFA depletion in Day 5 enriched *Artemia*. When the dietary contribution of AA, EPA and DHA to Day 5 *Artemia* was prominent (OWL+OIL - 8.9, 11.7, 7.5%, respectively; A1 DHA Selco 1.7, 5.3, 20.7%, respectively), starvation caused a reduction in their percentage contribution, and in particular, the contribution of the essential fatty acid DHA.

The quantitative changes in the EFA (Fig. 4.2 and 4.3) largely mirrored the results of the percentage data (Tables 4.3-4.5). An exception was evident in the essential fatty acid DHA in the A1 DHA Selco treatment, where Day 2 and Day 5 starved *Artemia* lost 22% ($P < 0.05$, Fig 4.2) and 20% ($P = 0.07$, Fig 4.3) respectively, compared to pre-starvation levels.

Other fatty acids reduced by starvation (Tables 4.3-4.5) included 18:1n-7 and 18:2n-6 while the saturated fatty acids (SFA) 16:0 and 18:0 underwent a degree of sparing with starvation. The large contribution of 18:2n-6 to the diets and subsequently the *Artemia* is reflected in low n-3/n-6 ratios. In particular, the n-3/n-6 ratio in the OWL and OWL+OIL fed animals was in the range of 0.1-0.7 and 0.3-1.0, respectively, compared to the target phyllosoma ratio of 2.7.

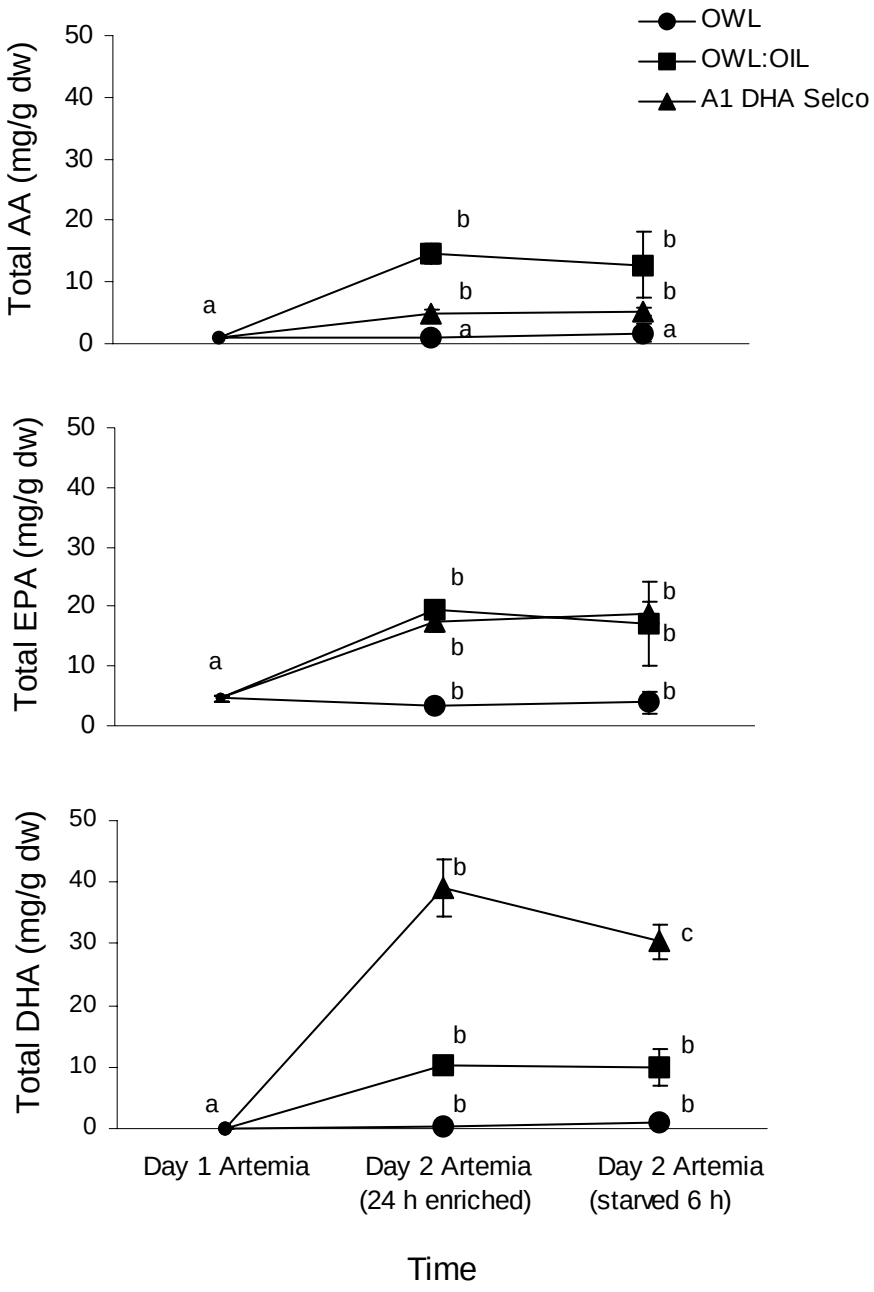

Fig. 4.2. Changes in the quantitative contribution of arachidonic (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to the total lipid in Day 1 *Artemia*, after 24 h enrichment (Day 2) and a further 6 h starvation. Different superscripts denote significant differences within a dietary treatment over time.

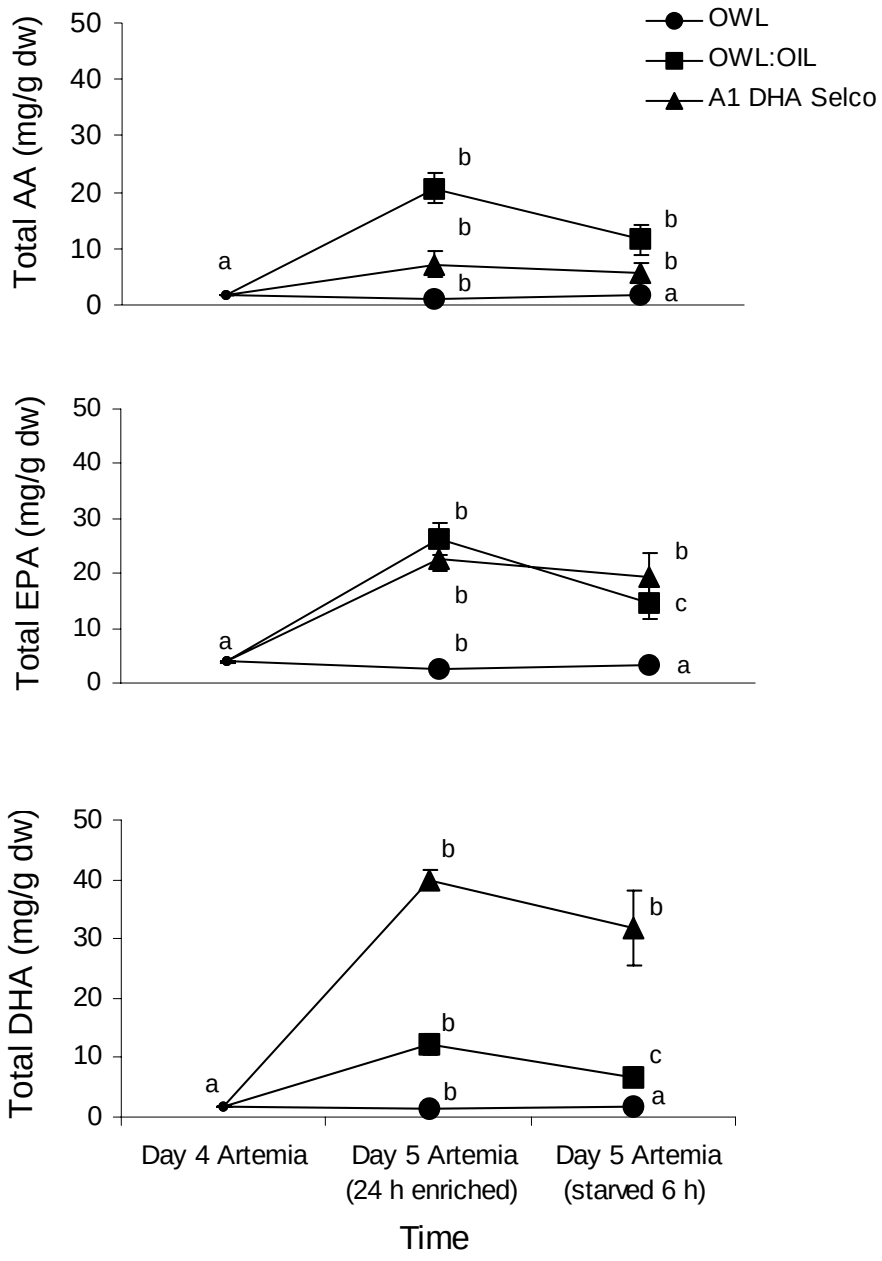

Fig. 4.3. Changes in the quantitative contribution of arachidonic (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) to the total lipid in Day 4 *Artemia*, after 24 h enrichment (Day 5) and a further 6 h starvation. Different superscripts denote significant differences within a dietary treatment over time.

4.6. Discussion

4.6.1. Gut evacuation trial

Even though enrichment of *Artemia* is widely used in aquaculture, it has to date been unclear what proportion of the enrichment is incorporated into body tissue and how much remains resident in the gut. The gut evacuation trial demonstrated that the gut content of juvenile *Artemia* (5 day old) at 3 h and 6 h made only a minor contribution to the total lipid and fatty acid profiles of the *Artemia*. While minimal gut retention times were not monitored in this trial, we have previously recorded a gut retention time of 7 min for actively feeding juvenile *Artemia* (Smith, unpublished). This is intermediate to retention times recorded for nauplii and adult *Artemia* of 10 and 3 min, respectively (Dobbeleir et al., 1980). While retention times in juvenile *Artemia* of up to 30 min have been recorded using starch to replace gut content (Nimura, 1989) we considered this time period is a measure of the time required to flush the alimentary canal of a particular material rather than gut passage time. In our trial, flushing of gut coloration in *Artemia* using beads was not achieved by 3 h and completed by 6 h. The reduction in gut coloration was thought to be due to sequential loss of bulk food content and then associated pigmentation from the gut lining, it is thought the time required for flushing differed from Nimura (1989) due to the different materials used for gut evacuation. In the w/o beads *Artemia* samples, there was no visible difference between the gut content prior to and after 6 h starvation with minimal feces present in the water column. *Artemia* are continuous, non-selective filter feeders (Provasoli and Shiraishi, 1959; Sorgeloos et al., 1998), so it appears that without the intake of suitable sized particulate matter the normal processes to stimulate gut evacuation do not occur. Material is maintained in the gut for at least 6 h when *Artemia* are held at 28ºC in clear water with no particulate matter present.

4.6.2. Juvenile Artemia - lipid and fatty acid composition

Artemia in the w/o beads treatment still had food remaining in their gut for at least 6 h after the commencement of the trial, i.e., after feeding was ceased. It is

probable that this prolonged gut resident time would allow the extraction of additional lipid from the food than otherwise would have occurred in actively feeding *Artemia*. As there was no difference in the amount of lipid between evacuated *Artemia* (w/beads) and starved *Artemia* (w/o beads) at 3 h, we propose that the majority of the lipid was removed by 3 h with resident material in the gut devoid of lipids. Therefore, any loss of gut content through predator feeding behavior, as often occurs when *Artemia* are fed to spiny lobster phyllosoma, should only alter the total *Artemia* lipid profile within a short period of the commencement of feeding, i.e. < 3 h, after which time the remaining lipid in the gut content can be considered as largely not contributing to the juvenile *Artemia* lipid profile. We found, as did Léger et al. (1986, 1987) that the fatty acid profile of unenriched *Artemia* contains only small amounts of the essential fatty acids AA, EPA and DHA (see Table 1). This finding highlights the need for a suitable source of enrichment if *Artemia* were to match the observed lipid profiles for spiny lobster phyllosoma (Smith, 1999; Phleger et al., 2001).

4.6.3. Artemia enrichment experiment - lipid and fatty acid composition

The 24 h enrichment trial reinforced the minor role that the gut content had on the total lipid or fatty acid profile of Day 5 *Artemia*. The result of minimal influence of gut content on lipid or fatty acid profiles was confirmed for Day 2 *Artemia* when sampled 6 h later. The enrichment diets used in the 24 h enrichment trial differed substantially from each other in both the level and type of lipid inclusion. Prior to enrichment, PL was the dominant lipid class in both Day 1 and Day 4 *Artemia*. However, as seen in other trials after enrichment, TAG became the major lipid class (McEvoy et al., 1996), even in *Artemia* fed a PL rich diet (OWL). This is probably due to *Artemia* taking up lipid in excess to requirements and storing it as TAG, a readily available energy source (Wickins et al., 1995). The efficiency at which lipids were assimilated varied greatly between diets. A1 DHA Selco is composed of 806 mg g^{-1} lipid dw, 4.9 and 2.8 times greater than the lipid content of the OWL and OWL+OIL diets, respectively. While A1 DHA Selco provided significantly greater lipid inclusion to Day 2 *Artemia* than did the enrichments of OWL and OWL+OIL, this pattern was not repeated for larger juvenile *Artemia*. There was no

significant difference between the lipid dw of Day 5 *Artemia* enriched with either A1 DHA Selco or OWL+ OIL regardless of whether the enrichment contained a high lipid content (A1 DHA Selco, 81% lipid) compared to one with a moderate level of lipid (OWL+ OIL, 29% lipid). This absence of a difference suggests that juvenile *Artemia* have a maximum lipid uptake during the 24 h enrichment period. In juvenile *Artemia*, the low uptake rate of enrichment products that are high in lipid suggests that increasing the density of *Artemia* in the enrichment diet, or reducing the application rate of the lipid emulsion would not appreciably alter lipid uptake.

Starvation for 6 h at 28ºC in this study resulted in no observable difference in lipid content in 2 or 5 day old *Artemia*, although there were significant changes in fatty acids. This contrasts with the findings of Evjemo et al. (2001) who found that *Artemia* nauplii lost 34% of their lipid content during 24 h starvation at 26ºC, while at 12ºC lipid losses during starvation were reduced to 11%. We suggest that during the initial 6 h of starvation lipid was conserved at the expense of another metabolic substrate, possibly carbohydrate, which has been demonstrated as important energy source in juvenile and adult *Artemia* (D'Agostino, 1980). A reduction in the abundance of some fatty acids such as, AA and EPA in the OWL+OIL treatment suggest they were supplied to *Artemia* in excess of their requirement, or as in the case of DHA whereby *Artemia* find it difficult to maintain as a storage product (Evjemo et al., 2001). As it is the intention to feed enriched juvenile *Artemia* to the predator spiny lobster phyllosoma at 18ºC (Ritar, 2001) the loss of lipids in enriched juvenile *Artemia* during a 20 h tank resident time would be low. Our results suggest that while there would be a decline in the essential fatty acid DHA over time in juvenile *Artemia*, it would occur at a marginally slower rate than if Day 2 metanauplii *Artemia* (this trial) or nauplii (Evjemo et al., 2001) were used. The large contribution of 18:2n-6 to the total fatty acid profile of both diet and enriched *Artemia* resulted in a low n-3/n-6 ratio, particularly in the OWL and to a lesser extent the OWL+OIL fed animals. We suggest that feeding these *Artemia* may result in the transfer of a low n-3/n-6 ratio to the predator species. It has been postulated, particularly in fish, that n-3/n-6 ratios less than 1, as occurs in the OWL and OWL+OIL enriched *Artemia*, may increase larval susceptibility to stress (Sargent, 1995). By reducing the level of 18:2n-6 in the *Artemia* diet (hence in

Artemia), the n-3/n-6 ratio potentially could be increased. This would be at the expense of the potential benefits of 18:2n-6 being available as an energy source (D'Souza, 1998) or precursor for AA production (Sargent, 1995). However, this should not be considered a negative factor, as many species are unable to produce a sufficient amount of AA by elongation of 18:2n-6, and even if they do, often numerous other unwanted C_{20} and C_{22} by-products may be formed (Sargent, 1995).

The specific inclusion of AA as a dietary EFA was targeted at the putative requirements of spiny lobster phyllosoma but may be incorporated at different rates to suit the requirements of a number of other crustacean and fish species. Recent research on larval penaeids (*Penaeus japonicus, P. semisulcatus* and *P. monodon*) (D'Souza and Loneragan, 1999) and finfish (*Paralichythys olivaceus* and *Sparus aurata*) (Estévez et al., 1997; Koven et al., 2001) suggests benefits such as a reduction in stress related mortality and improved pigmentation when AA was incorporated into dietary regimes.

AA is available in the marine environment in relatively small amounts however it contributes up to 8% to the total fatty acid profile of spiny lobster phyllosoma (Smith, 1999; Phleger et al., 2001). While it is unusual for temperate and polar marine species to contain such high levels of AA (Sinclair et al., 1986), this is not so for benthic species (Nichols et al., 1998a,b; Dunstan et al., 1999). The inclusion of AA at levels approaching those observed in phyllosoma from a wild origin was obtained in *Artemia* enriched with OWL+OIL. However, the percentage inclusion of EPA may still be insufficient in *Artemia* enriched with this diet. It is the balance of n-3/n-6 fatty acids and in particular of EPA: AA ratio, which has been signaled as important in this and other larval crustaceans (Sargent, 1995; D'Souza and Loneragan, 1999; Smith, 1999; Phleger et al., 2001). In a number of other species, AA has been suggested as having a major role as a precursor of eicosanoids, the highly biologically active molecules linked to moulting and stress response (Lytle et al., 1990; Sargent, 1995). Eicosanoid production from AA (n-6 fatty acid) is modulated by EPA (n-3 fatty acid), and failure to supply these two EFAs in the appropriate balance may result in adverse biochemical responses when fed to the predator organisms (Sargent, 1995).

It is the ability of 24 h enriched *Artemia,* in particular at Day 5, to resemble the EFA profile of their dietary source that shows promise for further manipulation to

meet the needs of host crustacean and fish predators. Within a 24 h period, Day 5 *Artemia* fed the OWL+OIL and A1 DHA Selco enrichments mirrored the percentage dietary inclusion levels of both AA and EPA. The incorporation of DHA into *Artemia*, at Day 5 was markedly less than its level in the enrichment, however greater losses occurred in younger (Day 2) *Artemia*. Estévez et al. (1998) found that, of the EFAs, the incorporation rate of DHA was less during *Artemia* nauplii enrichment and the loss highest during starvation, a situation analogous to that found in Day 2 and 5 *Artemia* in this study. Lower DHA incorporation in juvenile *Artemia* compared to dietary inclusion levels may be associated with the inability of juveniles to preferentially assimilate DHA during 24 h enrichment. We consider that DHA did not undergo significant retro conversion to EPA, as often occurs in *Artemia* nauplii (Navarro et al., 1999; Evjemo et al., 2001), because EPA was incorporated at a rate equivalent to inclusion levels in both OWL+OIL and A1 DHA Selco diets and appeared to increase independently of DHA.

The uptake and maintenance efficiency of various fatty acids in *Artemia* during 24 h enrichment to a large degree dictates the level that they should be included in the enrichments to attain a desired profile. Both AA and EPA were incorporated and effectively maintained in juvenile *Artemia* at levels similar to their inclusion in the enrichments. However, to achieve a fatty acid profile similar to the desired target species (spiny lobster phyllosoma), the percentage inclusion of EPA would need to be increased in the OWL+ OIL enrichment.

4.7. Conclusion

It appears that juvenile *Artemia* require the intake of suitably sized particulate matter to enable gut evacuation to occur. While gut content was not noticeably voided within 6 h of the cessation of enrichment, we also found that at 3 h and 6 h the gut content did not make a significant contribution to the total lipid content or fatty acid profiles of juvenile *Artemia*. Therefore, any loss of gut content in juvenile *Artemia* as a result of predator feeding behavior is insignificant in terms of the lipid content or fatty acid composition shortly after the cessation of enrichment. Juvenile *Artemia*, a life stage seldom used in feeding regimes, demonstrated the

ability to assume the EFA profile of their dietary source, in particular the AA and EPA profiles and to a lesser degree, DHA. This is a trait that has considerable potential for use in species that have diverse and perhaps non-traditional EFA requirements. Further research on optimizing *Artemia* EPA: AA ratios, decreasing the level of 18:2n-6 and increasing the n-3/n-6 ratio to reflect the composition of spiny lobster phyllosoma, is being conducted.

4.8. Acknowledgments

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5. Chapter five - Phyllosoma diet (b)

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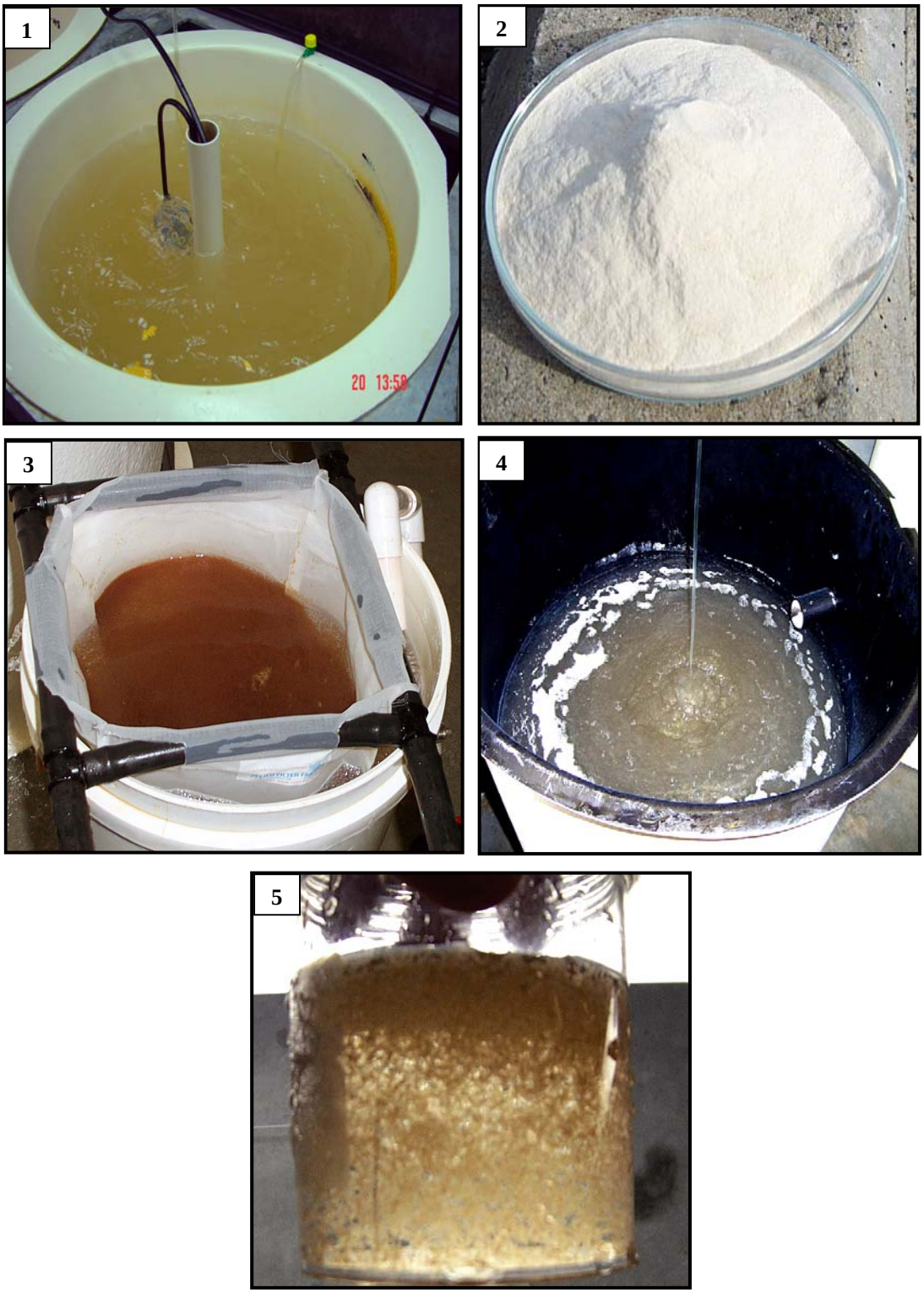

Plate 5. Enrichment of *Artemia* with a particulate form of ascorbic acid (ascorbyl-2-monophosphate; A2P). (1) Ongrowing *Artemia* to the desired size in culture vessels. (2) Particulate ascorbic acid; A2P. (3) Harvesting ongrown *Artemia* for enrichment. (4) Enrichment of *Artemia* with A2P. (5) Juvenile *Artemia* after 24 h enrichment with A2P.

5.1. Abstract

A study was conducted to establish whether a particulate form of ascorbic acid (AA), ascorbyl-2-phosphate (A2P), could be used to enrich *Artemia*. In the first experiment we examined the efficiency of A2P conversion to and maintenance of AA by juvenile *Artemia* (1.5 mm, 5 day old) held at 9 000 L^{-1} and 28°C for 24 h. Maximal uptake and assimilation was > 10 000 μg AA g^{-1} dw (representing > 1% *Artemia* dw) at enrichment rates of ≥ 1.2 g A2P L^{-1}. In the second experiment a similar biomass of instar II/III nauplii (1 mm, 2 day old) and juvenile (2.5 mm, 8 day old) *Artemia* were enriched for 6 or 24h at 28°C before starvation for 6 or 24 h at 18 or 28°C. At 0 h and after 6 and 24 h enrichment, AA levels were 485, 3468 and 11 080 μg g^{-1} dw in nauplii and 122, 4286 and 12 470 μg g^{-1} dw in juveniles. When *Artemia* nauplii or juveniles were enriched for 6 h and starved for 6 h at 18 or 28°C, there was no significant reduction in AA. Continuation of starvation to 24 h at 18 and 28°C reduced the level of AA to 3367 and 2482 μg g^{-1} dw in nauplii and 3068 and 2286 μg g^{-1} dw in juveniles. After 24 h enrichment, 6 h of starvation at 18 and 28°C reduced AA to 8847 and 7899 μg g^{-1} dw in nauplii and to 9053 and 8199 μg g^{-1} dw in juveniles. Continuation of starvation to 24 h at 18 and 28°C further reduced AA levels in nauplii to 6977 and 4078 μg g^{-1} dw and to 7583 and 5114 μg g^{-1} dw in juveniles. This study demonstrated that A2P could be assimilated as AA in the body tissue of different sized *Artemia* in a dose dependant manner and AA was depleted during starvation depending on time and temperature.

Keywords: *Artemia*, ascorbic acid, ascorbyl-2-phosphate, enrichment, starvation

5.2. Introduction

Established techniques to boost the endogenous ascorbic acid (AA) levels in *Artemia* nauplii have been demonstrated using AA/lipid emulsions (Merchie et al. 1995a). However, the transfer of the methodology to larger juveniles has not resulted in similar levels of enrichment (Lim et al. 2002). This is of particular interest to those involved in culturing species that utilize ongrown *Artemia* (Olsen et al. 1999; Lim et al. 2002; Smith et al. 2002; Ritar et al. in press).

Ascorbic acid is a water-soluble nutrient required by many aquaculture species (Magarelli et al. 1979; Sato et al. 1983; Dabrowski 1990,1992b). The essential nature of AA in fish and crustaceans can be traced to the absence of gulonolactone oxidase, an enzyme involved in the last step of AA synthesis from glucuronic acid (Dabrowski 1992a). For cultured fish and crustaceans the delivery of AA to juveniles and adults is generally facilitated by its incorporation into pellet feeds (Sandnes 1984, 1991; Soliman et al. 1986), while live feeds such as rotifers and *Artemia* are the vehicle of choice for larval stages (Merchie 1995a,b,c). L-ascorbic acid is the active form of AA, but its high degree of water solubility and associated degradation negates its use in aquaculture feeds. Water stable forms of AA are now available including a number of ascorbyl polyphosphate (ApP) derivatives used in pellet feeds, and ascorbyl palmitate (AP), an additive used in lipid enrichment emulsions for live larval feeds (Merchie et al. 1995a). Enrichment of *Artemia* with microalgae can also provide the benefits of increased endogenous AA levels (Dhont et al.1991; Naegel 1999; Ritar et al. in press)

In recent years the culture of new aquaculture species with life stage specific requirements has meant diversification in the use of *Artemia* to include live juvenile and adults as well as frozen or freeze dried *Artemia* biomass (Browdy et al. 1989; Dhert et al. 1992, 1993; Naessens et al. 1997; Olsen et al. 1999; Wouters et al. 1999; Lim et al. 2002; Smith et al. 2002). Traditionally there has been widespread use of *Artemia* nauplii in enrichment protocols due to their ready availability, ease of culture, acceptance by a range of larval species and the malleable nature of their biochemical composition (Sorgeloos et al. 1998). The ability of *Artemia* to metabolize and store specific biochemical substances (Watanabe et al. 1983; Léger et al. 1987; McEvoy et al. 1996; Evjemo et al. 1997; Estévez et al. 1998; Sorgeloos

et al. 1998; Narciso et al. 1999; Smith et al. 2002) while suffering no obvious detrimental physiological effects makes them an ideal vehicle for the delivery of chemotherapeutics, possibly including AA.

The basal AA requirement for many commercially important aquaculture fish and crustacean species is generally < 120 μg g^{-1} dry weight (dw), and may be as low as 20 μg g^{-1} dw during juvenile and adult stages (Sandnes et al. 1992; He and Lawrence 1993; Shiau and Hsu 1994; Boonyaratpalin et al. 1995; Giri et al. 1995). By ensuring that these basal AA requirements are met, deficiency symptoms such as lordosis and scoliosis (skeletal deformities) in fish (Dabrowski 1990), and black spot (melanised lesions under the exoskeleton) in crustaceans (Hunter et al. 1979; Magarelli et al. 1979) may be prevented. In some species, supplementing mega doses of dietary AA (up to 5000 μg g^{-1}) has reduced the impact of environmental stress caused by handling, crowding, poor water quality (hypoxia, metal toxicity) (Soliman & Viteri 1982; Thomas 1984), improved resistance to bacterial pathogens (Li & Lovell 1982; Liu et al. 1989) and physiological stresses involved in metamorphosis (Ishibashi et al. 1992; Merchie et al. 1996). Dabrowski (1992b) has extrapolated further suggesting that stress creates an increased AA requirement, with available AA reflecting survival potential.

It is a relatively simple process to deliver mega doses of AA to juvenile/adult fish and crustaceans using feed pellets fortified with ApP (Sandnes 1984, 1991; Soliman et al. 1986; Shiau & Hsu 1994). The provision of similar supplementation levels using *Artemia* boosted with AP/lipid emulsions or microalgae is more difficult to achieve (Dhont et al. 1991; Merchie et al. 1995b, 1996; Olsen et al. 2000; Lim et al. 2002). Merchie et al. (1995a) found that AP incorporated quickly as AA into *Artemia* nauplii during 24 h enrichment, increasing the endogenous levels from < 520 μg g^{-1} dw in unenriched *Artemia* to 1200 and 2500 μg g^{-1} dw with 10% and 20% AP/lipid emulsions, respectively. By contrast, Lim et al. (2002) enriched juvenile *Artemia* (5.0 mm, 10 day old) with 10 and 20% AP, but achieved low AA incorporation of 10.9 and 19.9 μg g^{-1} wet weight (ww), respectively.

The failure to boost the levels of AA in juvenile *Artemia* (Lim et al. 2002) to levels similar to that achieved with *Artemia* nauplii (Merchie et al. 1995 a,b,c) stimulated our research into the use of an alternate product. The novel enrichment of *Artemia* using ascorbyl 2-phosphate (A2P), a water stable ApP derivative

commonly used in pellet feed, allowed a number of questions to be examined. These included the:

- Uptake and incorporation of a particulate form (rather than an emulsion) of AA by *Artemia* of different sizes
- Ability of *Artemia* to metabolize A2P to AA
- Maximum AA levels attained by *Artemia* during short (6h) and long (24h) enrichment
- AA retention in *Artemia* during starvation at temperatures commonly used for aquaculture of temperate and tropical species.

A preliminary trial examined the dose rates required to achieve maximum tissue AA levels within 24 h. This rate was subsequently used to enrich *Artemia* (1.0 mm instar II/III nauplii and 2.5 mm juveniles) for 6 and 24 h at 28°C followed by starvation for 6 and 24 h at 18 and 28°C.

5.3. Materials and Methods

5.3.1. Artemia production

Decapsulated *Artemia* cysts (INVE, Great Salt Lake Prime Gold) were hatched in 50 L white fiberglass cones. Hatching, culture and subsequent enrichment of *Artemia* were conducted in aerated 1 µm filtered seawater maintained at 34 ± 1‰ and 28 ± 1°C. At 24 h, newly hatched *Artemia* nauplii were removed from the hatching cones, rinsed in freshwater for 2 min and cultured at a density of 5 ml^{-1} in 800 L conical tanks containing seawater. *Artemia* were ongrown to 1.0 mm (instar II/III, 2 days), 1.5 mm (early juvenile, 5 days) or 2.5 mm (late juvenile, 8 days) on a blended brine shrimp food containing primarily rice pollard, soy and wheat flour (Eyre Peninsula Aquafeeds Pty Ltd., South Australia). *Artemia* diet was added to the culture water three times daily at a rate to maintain a Secchi depth of 25–30 cm.

5.3.2. Experiment 1: Ascorbic acid dose rate

The enrichment product A2P is primarily a L-ascorbyl 2-monophosphate particulate form of AA, practically insoluble in water, containing a minimum of 35% AA activity; with a minimum 33% in the monophosphate form (Stay C35, Roche Vitamins Australia Ltd., Sydney). In a preliminary experiment to establish the optimal A2P dose rate, 945 000 x 1.5 mm juvenile *Artemia* were harvested from a 800 L culture vessel onto a 250 μm nylon screen and partitioned equally into 7 treatments in triplicate (45 000/replicate). All treatments were conducted in plastic vessels holding 5 L of aerated seawater. Treatments consisted of graduated amounts of A2P (0, 0.2, 0.4, 0.6, 0.9, 1.2 and 1.5 g L^{-1}) suspended in the water column. The A2P ration was divided into two equal portions and given at times 0 and 12 h from the commencement of the trial. To ensure the presentation of appropriate particle size for *Artemia* (Dobbeleir et al. 1980), A2P was blended for 30 sec in seawater (household blender, Sunbeam, Australia) before being passed through a 40 μm screen and added to the enrichment vessel. Successive washes and rubbing your hand around the inside of the screen were required to reduce all particles to < 40 μm and force them through the screen. At 24 h all treatments were terminated, *Artemia* were rinsed 3 times in 0.5 M ammonium formate, wrapped in aluminum foil, before storage in liquid nitrogen until sample analysis.

The uptake by *Artemia* of A2P from the water column, its efficiency of conversion (%) and storage as AA was measured as the change in total AA in *Artemia* after enrichment divided by the A2P added to the enrichment vessel x 100.

5.3.3. Experiment 2: Ascorbic acid assimilation and depletion

Experiment 2 examined the assimilation of A2P as AA in the body tissue of 1.0 mm *Artemia* nauplii and 2.5 mm *Artemia* juveniles after 6 and 24 h enrichment, and then the depletion of AA during starvation at 18 or 28°C for 6 or 24 h (Table 5.1).

Table 5.1 Ascorbic acid enrichment and starvation protocols for *Artemia* nauplii and juveniles, Exp2.

Animal stage	Nauplii (1mm)				Juveniles (2.5			
Enrichment	6h		24h		6h		24h	
Starvation 18°C	6	24	6	24	6h	24h	6h	24h
Starvation 28°C	6	24	6	24	6h	24h	6h	24h

Artemia were enriched in 20 L triplicate cones, containing 375 000 x 1.0 mm nauplii or 60 000 x 2.5 mm juveniles. The A2P enrichment was calculated at a rate of 1.2 g $L^{-1}d^{-1}$ (in 20 L), divided into two equal portions, and suspended in seawater before addition to the enrichment vessels at times 0 and 12 h. Triplicate samples of 1.0 mm *Artemia* nauplii (50 000) and 2.5 mm juveniles (8 000) were collected prior to enrichment (time 0), rinsed and stored for analysis as described above. The sampling and starvation regime of enriched *Artemia* involved removing half the volume of water (10 L) containing *Artemia* from each replicate after 6 h enrichment. From this subsample, the *Artemia* in 2 L were washed and stored for analysis as previously described. The remaining animals were rinsed with fresh water for 1 min on a 250 μm nylon screen, divided equally and resuspended into two vessels each containing 4 L of seawater maintained at either 18 or 28°C with immersion heaters. No enrichment was given to these *Artemia* from this stage forward. Starved *Artemia* in both temperature treatments were sampled at 6 and 24 h post starvation by removing 2 L of *Artemia* in seawater, rinsing and storing samples as previously described. The 10 L of *Artemia* in seawater remaining after the initial 6 h sub-sampling received further enrichment at 12 h; enrichment was terminated by rinsing and sub-sampling at 24 h. This was followed by 6 or 24 h starvation and sample collection as described above for post 6 h enrichment.

To maintain the same *Artemia* biomass at each size, calculations were based on the following individual *Artemia* size and dw: 1.0 mm – 2.95 μg, 1.5 mm – 6.05 μg and 2.5 mm – 17.66 μg and were derived from Abreu-Grobois et al. (1991),

Individual dw (μg) = $10^{(-2.53 + 1.63* \log(\text{len}) + 0.81* (\log(\text{len}) \times \log(\text{len})))} \times 10^3$.

Where len = length of *Artemia* from the anterior of the head to the anus.

5.3.4. Ascorbic acid analysis

Artemia samples were freeze-dried for 24 h (Dynavac freeze-drier F.D.3, -80°C, 33×10^{-3} Mbar), dw taken and 100 mg subsamples set aside for analysis. Ascorbic acid was extracted from the *Artemia* subsamples using metaphosphoric acid (3%) + acetic acid (8%) (MPA) by the method of Brown and Miller (1992), with minor modifications to the sonication step (sonicated once only for 30 sec). Total AA (sum of ascorbic acid plus dehydroascorbic) is detected using this assay, not AA in

the form of A2P. However, a preliminary trial demonstrated that there was minimal additional AA present in *Artemia* post enrichment when using a phosphatase incubation step to convert A2P to AA (Wang et al. 1988). HPLC analysis was conducted on 50 µL samples using a Waters Model 600E liquid chromatograph system. The derivatized product was detected using a Waters Model 475 scanning fluorescence detector; the excitation maximum was set at 355 nm and emission maximum at 425 nm. The peak area was quantified using Waters Millennium software. The column used was a C18 Novapak, Waters; 3.9 x 150 mm, which was eluted isocratically with 80:20 (v/v) 0.08 M potassium dihydrogen phosphate (pH 7.8) and methanol, respectively, flow rate of 0.8 ml min^{-1}. Results are expressed as µg AA g^{-1} dw.

5.4. Statistical analyses

Statistical analyses were conducted using one and two-way analyses of variance with Tukey-Kramer HSD tests used for post-hoc comparison (Sokal & Rohlf, 1995). Arcsin√ transforms were performed on percentage data. $P < 0.05$ was considered significantly different. Data are presented as mean ± sem. Statistics were executed using JMP version 3.1 (SAS Institute Inc.).

5.5. Results

5.5.1. Ascorbic acid dose rate

The endogenous level of AA in unenriched 1.5 mm juvenile *Artemia* (5 d old) was 165 μg g^{-1} dw. Enrichment of *Artemia* (9 000 L^{-1}) with A2P for 24 h resulted in significant increases in these levels (Fig. 5.1). Maximum enrichment was attained using A2P at a dose rate of 1.5 g L^{-1} (10 290 μg g^{-1} dw), however this level was not significantly greater (ANOVA) than attained with 1.2 g L^{-1} (10 170 μg g^{-1} dw). Use of A2P at a rate ≥ 1.2 g L^{-1} resulted in excess of 60-fold increases to *Artemia* AA levels, to > 1% dw. The uptake and conversion efficiency (%) at which A2P was removed from the enrichment medium and incorporated into *Artemia* as an active form of L-ascorbic acid varied from a low of 0.017% with the addition of 0.2 g L^{-1} to a maximum efficiency of 0.045% at 1.2 g L^{-1} (Fig. 5.2). The addition of A2P at a dose rate less than 1.2 g L^{-1} (0.2, 0.4, 0.6 and 0.9 g L^{-1}) resulted in lower AA uptake efficiencies by *Artemia*, as did increasing A2P to greater than 1.2 g L^{-1}.

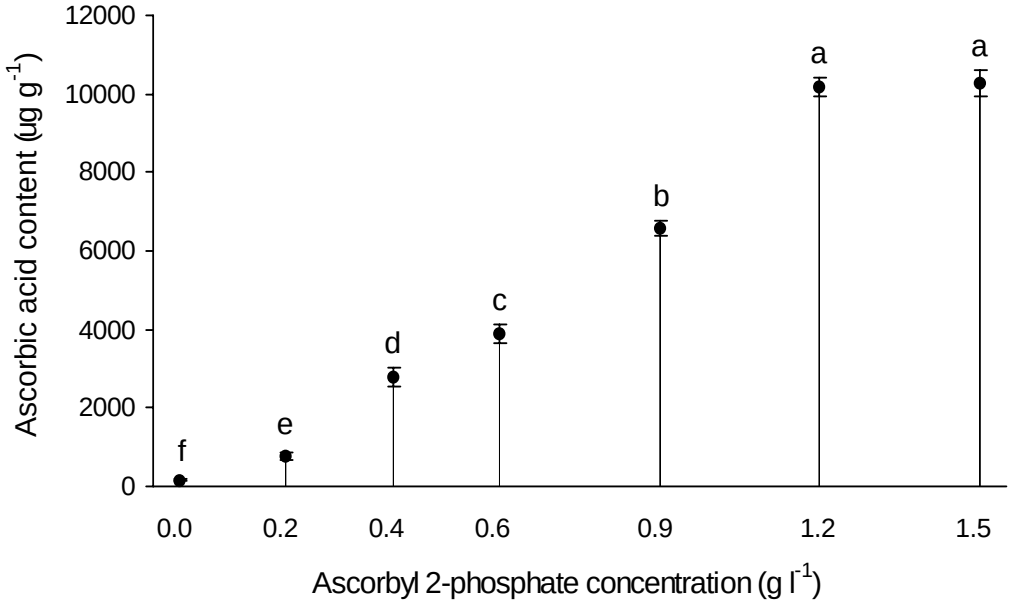

Fig. 5.1 Uptake and conversion of graduated amounts of ascorbyl 2-phosphate (A2P) g L^{-1} to L-ascorbic acid (AA, μg g^{-1} dw) by 1.5 mm juvenile *Artemia*. Different letters denote a significant difference between *Artemia* AA concentrations (ANOVA, $P<0.05$), n=3.

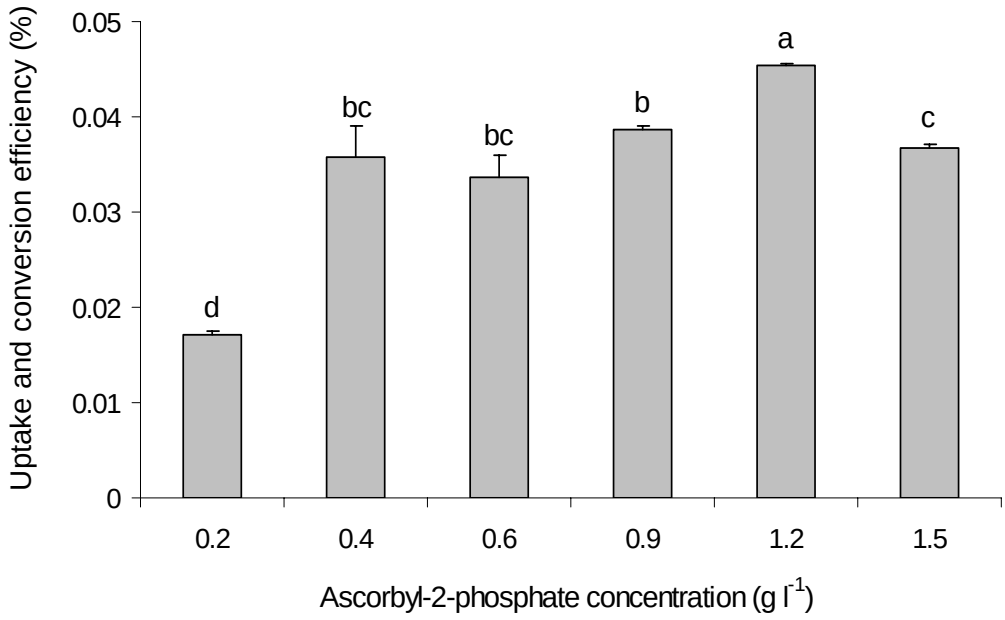

Fig. 5.2 The uptake and conversion efficiency (%) of ascorbyl 2-phosphate (A2P) to L-ascorbic acid (AA) by 1.5 mm juvenile *Artemia* after 24 h enrichment. Different letters denote a significant difference between AA efficiencies (ANOVA, $P<0.05$), n=3.

5.5.2. Ascorbic acid uptake and maintenance

The endogenous levels of AA in 1.0 mm and 2.5 mm unenriched *Artemia* were 485 and 122 μg g^{-1} dw, respectively. Both 1.0 and 2.5 mm *Artemia* were able to remove A2P, provided at a daily dose rate of 1.2 g L^{-1} from the water column, convert and store it as AA (Fig. 5.3a,b). The concentration of AA in 1.0 mm *Artemia* increased 7-fold to 3 468 μg g^{-1} in response to short-term enrichment (6 h). Following 6 h enrichment there was a significant interaction between starvation time and temperature ($F_2 = 22.15$, $P < 0.001$) on *Artemia* AA levels. A 6 h period of starvation at either 18 or 28°C had no significant effect on AA levels, with *Artemia* at 18°C even showing a small but non-significant increase (2%). Starvation for a further 18 h saw no reduction in the AA levels in *Artemia* nauplii at 18°C but there was a significant reduction in AA in animals held at 28°C, to 2482 μg g^{-1} dw an average depletion rate of 1.6 % h^{-1}.

Long-term enrichment (24 h) of 1 mm *Artemia* produced a 23-fold increase in AA levels from 485 to 11 080 μg g^{-1} dw (Fig. 5.3a). The loss in AA during

starvation was significantly affected by an interaction between starvation time and temperature (F_2 = 15.39, P = 0.002). A 6 h starvation period resulted in significant losses at 18 and 28°C, although there was no difference between temperature treatments, with AA depleted at a rate of 3.3% h^{-1} and 4.8% h^{-1}, resulting in AA levels of 8847 and 7899 μg g^{-1} dw, respectively. A further 18 h starvation saw a slowing in the average depletion rate to 0.9 % h^{-1} and 1.9 % h^{-1} resulting in 6977 and 4078 μg g^{-1} dw at 18 and 28°C, respectively.

Short-term enrichment (6 h) of 2.5 mm juvenile *Artemia* increased the endogenous AA levels 35-fold from 122 to 4 286 μg g^{-1} dw (Fig. 5.3b). A 6 h starvation period did not significantly reduce the level of AA in *Artemia* juveniles. Continuation of the starvation period for a further 18 h produced significant reductions of 28% and 47% resulting in AA levels of 3068 and 2286 μg g^{-1} dw in animals held at 18 and 28°C.

Long-term enrichment (24 h) of 2.5 mm *Artemia* resulted in a 102-fold increase in endogenous AA from 122 to 12 470 μg g^{-1} dw. The loss in AA during starvation was significantly affected by an interaction between starvation time and temperature (F_2 = 11.40, P = 0.006) and was similar to that experienced by 1.0 mm *Artemia* nauplii (Fig. 5.3a).

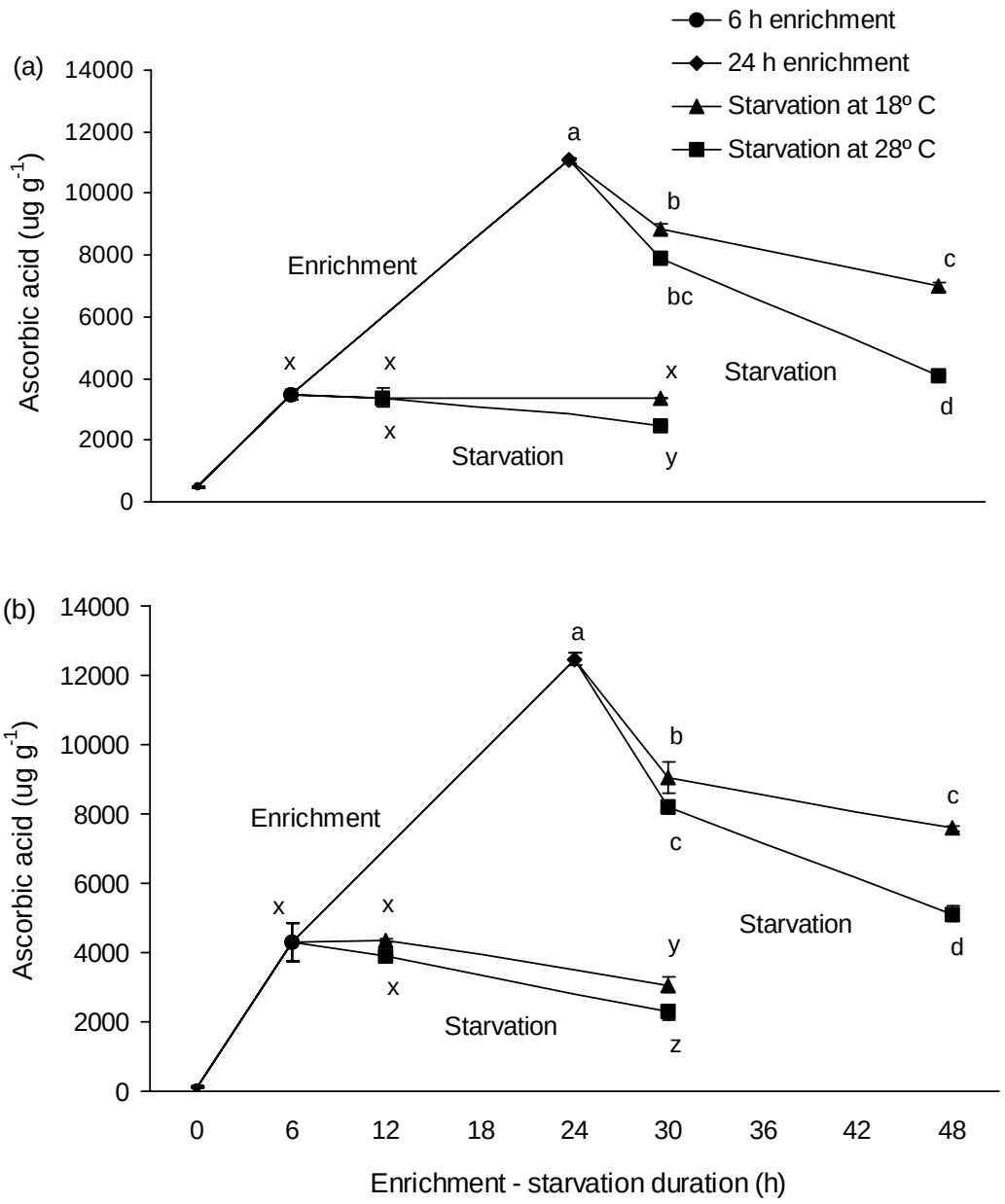

Fig. 5.3 Uptake, conversion and maintenance of ascorbyl 2-phosphate (A2P) to L-ascorbic acid (AA) during 6 and 24 h enrichment at 28°C, and 6 and 24 h starvation at 18 and 28°C. Two *Artemia* size class were examined, (a) 1.0 mm, 2 day old instar II/III nauplii and (b) 2.5 mm, 8 day old juveniles. Different letters denote a significant difference in AA retention post enrichment between 18 and 28°C at either 6 or 24 h within size class (two-way ANOVA, $P<0.05$), n=3.

During the first 6 h, there were rapid reductions in the amount of AA present in animals incubated at 18 and 28°C with losses of 4.5% h^{-1} and 5.7% h^{-1}, resulting in AA levels of 9053 and 8199 μg g^{-1} dw. A further 18 h starvation at 18 and 28°C saw a reduction in the loss rate to 0.7 % h^{-1} and 1.4 % h^{-1}, resulting in AA levels of 7583 and 5114 μg g^{-1} dw.

5.6. Discussion

Endogenous stores of AA in newly hatched *Artemia* have their origin in the geographical region, strain and nutrition of the parent broodstock (Sorgeloos et al. 1998). However, upon the commencement of feeding post-hatch, the *Artemia* diet becomes the major factor influencing biochemical composition and can be markedly influenced by enrichment protocols (Merchie et al. 1995a). AA has previously been delivered to prey items via *Artemia* nauplii using AP/lipid emulsions (Merchie et al. 1995a) or algal enrichment (Dhont et al. 1991; Naegel 1999). During our study, we demonstrated that A2P, like AP (Merchie et al. 1995a), was quickly assimilated by a range of *Artemia* sizes as the active form of AA, which is readily available to predator larvae (Sorgeloos et al. 1998). The maximum level of AA attained by 1.5 mm *Artemia* within a 24 h period was achieved with a dose rate of 1.2 g A2P $L^{-1}d^{-1}$, which is less than the calculated maximum ingestion rate ($i.r._{(max)}$) of 2.0 g A2P $L^{-1}d^{-1}$ (Abreu-Grobois et al. 1991) where; $i.r._{(max)} = 609.5 \times (1 - e^{(-0.075 \times dw)})$ μg d^{-1}, and dw is the individual *Artemia* dw (μg).

For the optimum dose rate to be substantially less than the calculated $i.r._{(max)}$ suggests that filtering capacity is not the limiting factor but rather the ability of *Artemia* to assimilate A2P in the form of AA within a limited time frame.

Prior to enrichment, instar II-III *Artemia* had endogenous AA levels similar to that reported by Merchie et al. (1995a). However, with increasing *Artemia* size (juvenile) lower pre-enrichment levels of AA were evident. While we are unaware of reports of endogenous AA in comparatively size unenriched *Artemia*, Lim et al. (2002) reported 5 mm, 10 d old *Artemia* had undetectable amounts of AA. We

suggest that decreasing levels of AA with increasing *Artemia* size is a general trend, although likely to be closely related to the *Artemia* culture diet. Typically, culture diets are based on rice, soy, wheat or oatmeal products (Dobbeleir et al. 1980; Sorgeloos et al. 1980; Dhert et al. 1992, 1993; Naegel 1999; Lim et al. 2002; Smith et al. 2002), all poor sources of water-stable forms of AA. Ritar et al. (in press) reported AA levels of 1680, 960 and 240 μg g^{-1} dw in 0.8, 1.5 and 2.5 mm *Artemia* respectively, however this was only after enrichment with Isochrysis sp. (Tahitian strain) (T-ISO), an algae reported to be high in AA (Brown and Miller, 1992).

Merchie et al. (1995a) demonstrated uptake and maintenance of AA in instar II/III *Artemia* nauplii using a range of lipid emulsions containing up to 30% AP, but more commonly 20%, which resulted in *Artemia* AA levels of up to 2500 μg g^{-1}. *Artemia* that were enriched with A2P quickly assimilated it in the form of AA, and by 6 h had attained levels greater than observed by Merchie et al. (1995a) using AP for 24 h. In both our study and that of Merchie et al. (1995a), at this level of enrichment, a 6 h starvation period resulted in a minor net increase in the level of AA, possibly representing the conversion of remnant A2P (AP in the study of Merchie et al. (1995a)) in the *Artemia* gut metabolized to AA during starvation. Demonstrating that the complete metabolism of A2P to AA can take up to 6 h and provides a useful reservoir to maintain the short-term AA tissue content in *Artemia*, unlike lipid enrichment where the gut content had no effect on tissue lipid content within 3 h of the cessation of supplementation (Smith et al., 2002). The gut reservoir of A2P is present due to minimal gut voiding occurring in the absence of feeding stimuli (Smith et al. 2002). It is likely that this phenomenon was not obvious in 24 h enriched animals due to the natural depletion rate in *Artemia* being in excess of the gut content of unconverted A2P.

In this study, AA levels of > 12 000 μg g^{-1} dw were obtained in juvenile *Artemia* after 24 h enrichment compared with a maximum of 20 μg g^{-1} ww (equivalent to 200 - 300 μg g^{-1} dw) obtained by Lim et al. (2002). A number of factors may have led to these differences including low pre-enrichment AA levels, shorter enrichment duration and their use of a soluble AA enrichment product (AP). Dobbeleir et al. (1980) reported that juvenile and adult *Artemia* have a poor ability to ingest soluble products compared to nauplii, which effectively remove soluble and small particulate matter up to 30 μm in size. Juveniles and adult *Artemia* are predisposed

to removing particulate matter in a size range up to 50 μm, effectively starving when presented only with soluble products (Dobbeleir et al. 1980). Given this scenario, it is likely that *Artemia* nauplii could utilize an AP/lipid emulsion while juveniles or adults would have an impaired capacity to do so, suggesting that enrichment for larger juvenile *Artemia* is more effectively delivered via particulate presentation.

The ability to boost AA levels using 20% AP for instar II/III *Artemia* nauplii has been established (Merchie et al. 1995a). However, even for this size class of *Artemia* A2P enrichment may be beneficial by allowing the targeted delivery of AA mega doses (4.5 times higher than with 20% AP). Delivery may be tailored to suit stressful husbandry or culture requirements such as grading or metamorphosis (Sorgeloos et al. 1998), transport of ornamental fish (Lim et al. 2002), or in crustacean species where depletion of body stores is evident post-ecdysis (Merchie et al. 1997). Depletion of AA in crustaceans has been linked to fast collagen formation associated with growth during ecdysis events where AA is a co-factor in collagen formation (Hunter et al. 1979). The benefit of intermittent mega doses of AA to a prey organism will be dependent upon their capacity to metabolize and store additional AA within a short period prior to a stress event (Johnston et al. 1989). Thus the AA prophylactic treatment of predators, such as *Jasus edwardsii* a spiny lobster commonly exhibiting ecdysis related larval mortalities, (personal observation) may be possible using AA enriched *Artemia*, and juveniles in particular.

Artemia nauplii and juveniles have the ability to assimilate high levels of AA during 6 and 24 h enrichment, however it was only in *Artemia* enriched for the shorter period where AA depletion rates were minimal. This supports the finding of Merchie et al. (1995a), in which the AA levels in starved *Artemia* after enrichment were maintained at approximately 2 500 μg g^{-1} dw. *Artemia* enriched with mega doses of AA appear to have difficulty in maintaining levels in excess of 10 000 μg g^{-1} dw (equivalent to 1% body dw) without supplementary feeding when held at the temperatures of 18-28°C. Losses during starvation are synonymous to losses attained during feeding *Artemia* to predators in culture. If there is a requirement to maintain continuous mega doses of AA, appropriate feed protocols should be instigated. These may include feeding *Artemia* to predators in small doses often

during a 24 h period, rather than once daily, with AA losses minimized in surplus feed by storage of AA-enriched *Artemia* at low temperatures (Merchie et al. 1995a).

5.7. Conclusions

This study demonstrated the ability of A2P to boost the endogenous level of AA in *Artemia* nauplii and juveniles in terms of both enrichment level and time taken to achieve those levels. *Artemia* AA levels were shown to be very plastic, and demonstrate a dose-dependant response to enrichment while subject to time/temperature interactions during starvation. The use of A2P boosted *Artemia* may be useful for continual or targeted AA supplementation to predator marine larvae especially those that require live feeds and larger prey items.

5.8. Acknowledgments

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5.9. References

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6. Chapter six - Broodstock Supplement (a) - Lipids

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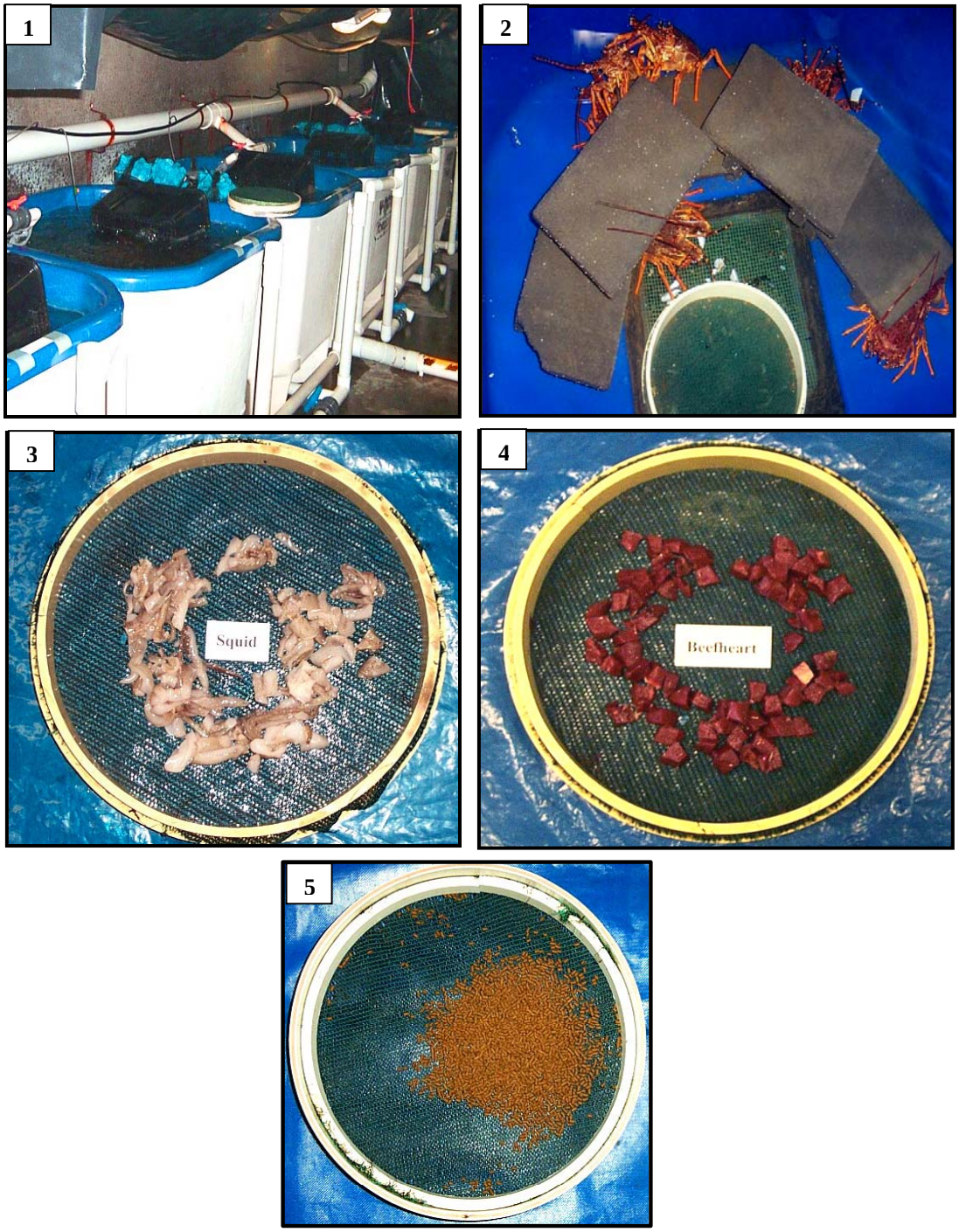

Plate 6. Experimental system and dietary components used in the broodstock lipid study. (1) Broodstock tanks. (2) Lobsters in hides. (3) Squid dietary component. (4) Beefheart dietary component. (5) Penaeid compound dietary component (pellet).

6.1. Abstract

Adult spiny lobster females were starved for a 3-week period then fed either a squid or beef-based diet for 5 months during ovarian maturation to ascertain the pattern of lipid and fatty acid usage, deposition and resilience to change in the digestive gland, ovary and tail muscle. Phyllosoma larvae resulting from these broodstock and animals from the wild were subject to an activity test (1 h exposure to temperature and salinity stresses) to ascertain their competency at hatch. Larval competency was validated with a period of culture. Prior to and during the feeding period broodstock lipid concentration was extremely high in the digestive gland (66-74% of tissue dry weight; dw), moderate in the ovary (38-43% dw) and low in the tail muscle (5-8% dw). The lipid profiles of the digestive gland, ovary and tail muscle were dominated by triacylglycerol (90%), polar lipid/triacylglycerol (52/43%) and polar lipid (90%), respectively. The digestive gland lipid content was reduced by starvation but increased by the end of the feeding period, suggesting this organ is used as an energy (lipid) reservoir. By the completion of the study, the fatty acid profile of the digestive gland closely resembled that of the diet. By contrast, the concentration of lipids and fatty acids in the ovary and tail muscle remained relatively stable independent of diet, although the ovary size and lipid content increased concomitant with maturation. Phyllosoma resulting from the broodstock dietary treatments and animals from the wild had similar lipid class profiles; polar lipids and sterols constituted over 98% of the total lipid. While their fatty acid profiles were similar, phyllosoma from wild broodstock had a lower content of all major fatty acids (except 20:4n-6) were larger, attained higher survival (up to Stage IV) and had lower inactivity counts (stress index). During this study the temperature and salinity parameters used in the activity test were modified to improve the predictive capacity of the test.

Keywords: broodstock, fatty acid, *Jasus edwardsii*, lipid, lobster, starvation.

6.2. Introduction

Spiny lobsters (Decapoda: Palinuridae) are opportunistic foragers; the nature of this feeding strategy exposes them to a wide variety of food items, which may be subject to seasonal or spatial availability (Jernakoff et al., 1993; Barkai et al., 1996). The natural diet of spiny lobsters is thought to be dominated by molluscs (mussels and barnacles), coralline algae and crustaceans, with minor dietary contributions from echinoids, worms, and seagrasses (Hickman, 1946; Barkai et al., 1996; Frusher et al., 1999). While spiny lobsters are selective foragers for particular dietary items (Fielder, 1965; Barkai et al., 1996), in environments where food is scarce, dietary intake is more likely to reflect food availability (Fielder, 1965; Edgar, 1990). This poses the question of spiny lobster sensitivity to fluctuations in dietary source and the impact that this may have upon the processes of broodstock maturation and subsequent larval competency.

In recent times, the pressure to increase lobster production from fully exploited fisheries has stimulated interest in their aquaculture potential (Schaap, 1997). One prominent candidate is *Jasus edwardsii* (Hutton), an ecologically significant spiny lobster which plays an important role in shaping temperate reef systems of southern Australia and New Zealand waters (Frusher et al., 1999). *J. edwardsii* is currently subject to propagation research in Japan (Kittaka, 1994), New Zealand (Tong et al., 2000) and Australia (Thomas et al., 1998).

For many cultured crustacean species the nutritional requirements of broodstock are poorly understood. As dietary regime is known to impact upon reproductive performance (Harrison, 1990; D'Abramo, 1997) the ability to maximise the benefits of nutritional input while minimising costs are a major concern for aquaculturists. The lipid profile of diets often cascades through broodstock into eggs and the larval profile (Watanabe, 1995), with the result that the larval profile often reflects that of the adult diet (Cahu et al., 1986, 1994; Narciso, 1999). This is of particular interest when it is considered that the uptake of highly unsaturated fatty acids (HUFA) by broodstock has been correlated with various aspects of reproductive performance and larval viability (Primavera et al., 1979; Abi-ayad et al., 1995).

In spiny lobsters, reproductive performance is measured using several parameters including sexual maturation, egg extrusion, fecundity, hatchability and

larval viability (MacDiarmid and Kittaka, 2000; Smith et al., 2002). The influence of diet on some of these parameters has been examined in a number of crustacean (Browdy et al., 1989; Wen et al., 2002) and fish species (Duray et al.,1994; Abi-ayad et al., 1995). In *J. edwardsii*, the relationship between dietary lipid and fatty acid abundance, in particular the influence of the essential fatty acids (EFA) 20:4n-6 (arachidonic acid, ARA), 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) on ovarian maturation and larval competency is unknown.

An activity test to instantaneously evaluate larval competency at hatch was developed for this species (Smith et al., 2003b). The test was further refined and tested here to examine the effects of maternal nutrition on offspring viability.

This study had three objectives:

1. To investigate the effects of broodstock diet on the lipid and fatty acid profiles of the digestive gland, ovary and tail muscle of broodstock prior to egg extrusion and on larvae at hatch.
2. To ascertain the pattern of organ lipid storage and sequestering during starvation and during ovarian maturation.
3. To assess the effect of broodstock diet on larval competency while refining the activity test for the diagnostic selection of competent larvae.

6.3. Materials and Methods

6.3.1. Broodstock collection and holding

Experimental work was carried out at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Hobart, Australia. Captive broodstock from Tasmania's west coast (43° 19' S, 145° 52' E) were held at the facility for 7 months prior to the feeding trial in January 2001. Phyllosoma from wild ovigerous broodstock caught from the same location in June 2001 were used to compare lipid and fatty acid profiles, larval size, as well as survival in activity tests and culture with those from captive broodstock. All broodstock were held under ambient

conditions of temperature and photoperiod. Temperature gradients ranged from 9.5°C in winter to 18.0°C in summer. Lobsters were exposed to a light intensity of < 4 µmol s^{-1} m^{-2} during daylight hours.

Captive animals were weighed (603.8 g ± 5.7 g) and measured (carapace length, 106.4 ± 0.4 mm), with 10 animals (3 males, 7 females) randomly distributed to each of 6 x 600 l square fiberglass tanks. Flow-through seawater was provided at 600 l h^{-1}, facilitating 100% water volume exchange every 1 h. Each tank had 400mm lengths of 100mm diameter PVC pipe for shelter. The wild-caught ovigerous broodstock were similar in weight (609 ± 3.1 g) and carapace length (105.2 ± 0.1 mm) to captive stocks, minimizing any possible broodstock effects on organ size or phyllosoma competency. Before commencing the dietary study, animals were starved for 3 weeks to minimize the residual effects of storage products on their lipid and fatty acid profiles and to assess the fate of those stores during starvation.

6.3.2. Broodstock dietary regime

The two experimental diets that were used during this study were done so primarily because of their palatability to lobsters, which ensured the maximum uptake of nutrients and secondly, their vastly different EFA profiles, they were:

- Diet SP - squid (*Nototodarus sloanii*) and commercial prawn pellet (Vital prawn, Higashimaru, Japan) (4:1 dry weight – dw, high in EPA and DHA)
- Diet BP - beefheart (pasture-fed cattle) and commercial prawn pellet (4:1 dw, high in ARA).

Broodstock were fed to excess three times per week from February 2001 for 5 months until mating and egg extrusion had occurred. Prior to the commencement of the study, captive broodstock were fed mussels (*Mytilus edulis*), squid and commercial prawn pellets (2:2:1). To counter selective consumption of particular feed components (Fielder, 1965; Barkai et al., 1996) different dietary components were fed on separate days, lobsters were observed to feed daily. For Diet SP, squid was fed on Friday and Monday with pellets fed on Wednesday; for Diet BP, beefheart was fed on Friday and Monday with pellets fed on Wednesday. Feed was placed on trays located on the tank bottom in the evening with any uneaten food

removed within 24 h. Ration size was reduced from 2% of biomass per week in summer to 1.5% during winter (dw diet : wet weight (ww) lobster) and was determined by monitoring lobster weights and consumption patterns. A small amount of excess diet remained post-24 h feeding.

Representative samples of broodstock dietary components (10g ww) were collected for lipid and fatty acid analysis at the commencement of feeding and 1, 3 and 5 months after feeding had commenced.

6.3.3. Broodstock sampling and monitoring to phyllosoma hatch

Triplicate samples of female broodstock from each dietary treatment were killed and tissue samples (digestive gland, ovary and tail muscle) were taken for lipid and fatty acid analysis. Samples were taken prior to the commencement of the study, after 3 weeks starvation and 1, 3 and 5 months after the commencement of feeding. A period of starvation would deplete lipid fatty and acid stores and hence provide an insight into the mobilization and utilization of these products. Due to the long lead-time between starvation and egg extrusion (5 months) and the inherent ability of spiny lobsters to adjust to seasonal changes in food source and availability (Jernakoff et al., 1993; Barkai et al., 1996) it was considered that there would be no undue effects on the processes of maturation. Prior to dissection, broodstock were placed in an ice slurry for 1 h to induce a chill coma. Ovarian stage of development was recorded, with 7 recognized macroscopic stages differentiated primarily on size and colour (Fielder, 1964). Two indices were calculated for broodstock: the organ lipid index (organ lipid dw/total body ww x 100) and the gonadal somatic index (GSI, ovary ww/total body ww x 100).

Broodstock tanks were inspected daily for mortalities, molts and ovigerous females, while closer inspection of animals occurred during weekly tank cleaning. Broodstock from both dietary treatments experienced one death each during the trial. Animals of similar weight replaced mortalities but the replacement animals did not extrude eggs. Prior to hatching (2-7 days), females were removed from the dietary treatment tank and placed into individual 20 l hatching containers suspended in the broodstock hatching tank (Smith et al. 2003a). Briefly, the hatching container consisted of a 20 l plastic bucket fitted with a lid, a section of the lower portion of

the bucket has been removed and replaced with a 500 μm screen, phyllosoma larvae are retained within the container by the screen while water is exchanged at 1 l min^{-1}.

6.3.4. Phyllosoma assessments

At hatch, phyllosoma larvae were skimmed from the surface of the hatching container and allocated randomly to the following treatments:

1. culture of larvae for 42 d with length and survival measured at molt Stages I - IV;
2. Lethal Dose 50 (LD-50), the time (d) for 50% of unfed Stage I larvae to die;
3. survival of larvae in 1 h temperature and salinity challenges (activity test);
4. sample for analysis of lipids and fatty acids.

The application of salinity activity tests to crustacean larvae is a common method to test quality and is based on the premise that you are exposing animals to an adverse condition that they are able to respond to based on their competency (Tackaert et al., 1989; Villalón,, 1991; Clifford, 1992; Fegan, 1992; Bray and Lawrence, 1992; Samocha et al., 1998). The correlation between survival and any particular variable will be dependant upon how much that variable altered between treatments and its influence upon competency, it will not be a condition that they would normally be exposed to in the wild (Racotta et al., 2003). It is suggested that salinity stress tests measure a combination of factors ranging from an animals nutritional status to their ability to mobilize nutrients under duress, in particular, to supply energy for ATPase pumps during osmoregulatory stress, conditions expected during salinity challenges (Racotta et al., 2003).

Relative viable fecundity was measured from individual females at hatch. This is a measure of the total number of phyllosoma hatching off a female, divided by female carapace length (mm) (units – number of phyllosoma per mm of carapace length). It does not include non-viable eggs or animals that do not progress beyond the naupliosoma stage (a brief 0.5 – 1 h post-hatch stage).

6.3.5. Morphological measures

The cross-sectional diameter of twenty randomly chosen eggs was measured on the first day that phyllosoma began to hatch from females (a typical hatch period lasts 4-5 days per female). Additionally, total body length (anterior tip of the cephalothorax to the posterior point of the abdomen) was measured in twenty newly-hatched Stage I phyllosoma from each sample. Measures of total body length were again taken on days 14, 28 and 42 at Stages II, III and IV, respectively, as described by Lesser (1978). Measurements were obtained using an overhead projection microscope (Nikon profile projector, model 6C, 20 times magnification).

6.3.6. Larval rearing

Phyllosoma resulting from the Diet SP (n=6), Diet BP (n=9) and wild broodstock (n=6) were cultured to Stage IV (42 d) in lightly aerated 1 l glass beakers maintained in water baths at 18ºC. Additional triplicate groups of phyllosoma congenetics remained unfed in the same system and under the same conditions to assess their level of endogenous reserves and physiological condition, using a LD-50.

During Stage I, larval densities in both fed and unfed treatments were 100 larvae l^{-1}. Larval numbers were subsequently reduced to 50 and 30 larvae l^{-1} at Stages II and III, respectively. Larval rearing was conducted in 1 l of water except when low survival in previous stages resulted in volumes being reduced to maintain constant rearing densities.

Phyllosoma were fed 1.5mm juvenile *Artemia* at a rate of 3 ml^{-1} every day, following daily total water exchange in the beakers, flushing away uneaten *Artemia* and application of antibiotics (oxytetracycline hydrochloride 25 mg l^{-1}, Intervet Engemycin 100, Melbourne, Australia) to the culture water. Prior to use, *Artemia* were disinfected in a formalin bath (100 mg l^{-1} for 10 min) and rinsed on a 250 µm screen with fresh seawater.

6.3.7. Cumulative activity test

The activity test developed to ascertain larval competency (Smith et al. 2003b) was examined further using the stress parameters of 8, 10, 12‰ and 21, 23, 25°C. Briefly, phyllosoma from individual females (triplicates n=20 larvae) were placed in different combinations of salinity and temperature, and monitored for activity at 3-min intervals. Prostrate larvae were counted as 'inactive' and cumulative inactive totals (stress index) were tallied over 1 h. A large stress index indicated that animals had succumbed sooner to the stress parameters. Results were validated by relating stress indices with survival in culture or an LD-50, a low stress index or high LD-50 is indicative of higher survival of congenetics in culture.

6.3.8. Quantification of lipids and fatty acids

Feed, broodstock tissues and phyllosoma samples were quantitatively extracted for lipids using a modified Bligh and Dyer (1959) one-phase methanol/chloroform/water extraction (2:1:0.8, by vol.). Each sample was extracted overnight and the phases were separated the following day by addition of chloroform and water (final solvent ratio, 1:1:0.9, v/v/v, methanol/chloroform/water). The total solvent extract was concentrated (i.e. solvents removed *in vacuo*) using rotary evaporation at 40°C and lipid content determined gravimetrically. The proportions of the major lipid classes were determined by analyzing a portion of the total lipid extract (in triplicate) on Chromarod S-III silica rods with an Iatroscan Mk III TH-10 TLC-FID analyzer (Iatron Laboratories, Japan). The solvent system used was hexane/diethyl ether/acetic acid (60/17/0.2, v/v/v) (Volkman and Nichols, 1991).

Fatty acid methyl esters (FAME) were produced from an aliquot of the total solvent extract treated with methanol/4N hydrochloric acid/chloroform (10:1:1, by vol; 80°C, 2 h) (Christie, 1982). FAME were extracted into hexane/chloroform (4:1, v/v, 3 x 1.5 ml). Gas chromatographic (GC) analyses of FAME were performed with a Hewlett Packard 5890A GC equipped with a HP-5 cross-linked (5% phenyl-methylpolysiloxane) fused silica capillary column (50 m long, 0.32 mm i.d., 0.17um film thickness), a flame ionization detector (at 310°C), a split/splitless injector (at 290°C) and an HP 7673A auto injector. Helium was the carrier gas. Following

addition of tricosanoic acid methyl ester internal standard, samples were injected in splitless mode at an oven temperature of 50°C. After 1 min, the oven temperature was raised to 150°C at 30°C min^{-1}, then to 250°C at 2°C min^{-1} and finally to 300°C at 5°C min^{-1}, after which it was maintained isothermally for 15 minutes. Peaks were quantified with Waters Millennium software (Milford, MA, USA). Individual components were identified by comparing retention times with those obtained for authentic and laboratory standards, selected samples were analyzed by gas chromatograph-mass spectrometry (GC-MS) to verify specific FAME.

GC-MS analyses of the FAME were performed with a Finnigan GCQ Plus GC-MS System fitted with on-column injection set at 45°C. Samples were injected using an AS2000 autosampler into a retention gap attached to a HP 5 Ultra2 50m, 0.32 mm id, and 0.17.um film thickness column using helium for the carrier gas. Typical mass spectrometer conditions were as follows: EV, 70eV; Emission current 250, transfer line 310^{O}C, source temperature 240°C, 0.8 scans /sec, mass range 40-650 Dalton.

6.4. Statistical analyses

Statistical analyses were conducted using one and two-way analysis of variance (ANOVA) with Tukey-Kramer HSD tests used for post-hoc comparison. Arcsine√ transforms were performed on percentage data (Sokal and Rohlf 1995). Logistic regression and correlation analysis was used. Probabilities of < 0.05 were considered significantly different. Data are presented as mean ± standard error of the mean (sem). Statistics were executed using JMP version 5.1. (SAS Institute Inc., Cary, NC, USA).

6.5. Results

6.5.1. Dietary lipid and fatty acid profiles

The lipid profile of the dietary components squid, pellets and beefheart were distinct; squid contained primarily polar lipid (PL) and sterol, the pellets were dominated by PL and triacylglycerol (TAG), while beefheart contained the greatest amount of lipid, predominantly PL (Table 6.1). Incorporation of these components into their respective dietary ratios resulted in PL being the most abundant lipid class in both Diet SP and BP (69.9 and 94.5 mg g^{-1}, respectively), while sterol (9.8 mg g^{-1}) was of secondary abundance in Diet SP and TAG (18.1 mg g^{-1}) in Diet BP. Diet BP contained the greatest amount of lipid with 126.6 mg g^{-1} compared to Diet SP with 91.1 mg g^{-1}.

The squid fatty acid profile was dominated by DHA, 16:0 and EPA, while pellets additionally contained two prominent monounsaturated fatty acids (MUFA), 18:1n-7 and 18:1n-9 (Table 6.1). Beefheart contained a high proportion of C 18 fatty acids and ARA with low levels of EPA and DHA. The dominant features highlighted by grouping fatty acids were the small amounts of MUFA present in squid, while beefheart had low EPA/ARA and DHA/EPA ratios. The fatty acid profiles of the SP and BP diets were similar to the squid and beef profiles, respectively. The polyunsaturated fatty acids (PUFA) accounted for approximately half of the total fatty acids of Diet SP, and while present in Diet BP in a similar amount (on a dw basis), it accounted for less than a third of the total fatty acids. The pellet component of Diet BP supplied small amounts of DHA (0.3 mg g^{-1}) and EPA (2.9 mg g^{-1}), whereas the beefheart component supplied even less although providing an elevated content of ARA (5.1 mg g^{-1}), and resulted in low DHA/EPA and EPA/ARA ratios compared to Diet SP.

Table 6.1 Quantitative fatty acid profiles of dietary ingredients and calculated combined diets fed to broodstock. Diet SP consisted of squid: pellet (4:1) and Diet BP beefheart: pellet (4:1). Data are presented as mean ± sem (n=3).

	Dietary ingredient (mg g^{-1} dw)			Diets (mg g^{-1} dw)	
	Squid	Beefheart	Pellet	Diet SP	Diet BP
Lipid Class					
Triacylglycerol	0.3 ± 0.2	13.8 ± 0.1	35.5 ± 0.3	7.3 ± 0.2	18.1 ± 0.1
Free fatty acid	1.5 ± 0.4	5.0 ± 0.0	6.5 ± 0.2	2.5 ± 0.4	5.3 ± 0.0
Sterol	10.5 ± 0.7	6.9 ± 0.2	6.8 ± 0.1	9.8 ± 0.7	6.9 ± 0.1
Polar lipid	76.1 ± 6.7	106.8 ± 0.1	45.1 ± 1.1	69.9 ± 4.2	94.5 ± 0.9
Other lipids[1]	1.1 ± 0.2	0.5 ± 0.1	3.9 ± 0.1	1.6 ± 0.1	1.3 ± 0.1
Total lipids	89.5 ± 0.4	133.9 ± 1.2	97.6 ± 0.6	91.1 ± 0.4	126.6 ± 0.6
Fatty Acids					
14:0	1.2 ± 0.3	0.7 ± 0.1	3.6 ± 0.1	1.7 ± 0.3	1.3 ± 0.1
16:1n-7	0.2 ± 0.0	1.9 ± 0.0	5.6 ± 0.3	1.3 ± 0.1	2.6 ± 0.1
16:0	17.4 ± 0.0	13.7 ± 0.4	20.7 ± 0.9	18.1 ± 0.2	15.1 ± 0.5
18:2n-6	0.2 ± 0.0	11.8 ± 0.2	2.4 ± 0.1	0.6 ± 0.0	9.9 ± 0.2
18:1n-9/3n-3[2]	1.2 ± 0.4	22.8 ± 0.4	12.5 ± 0.5	3.4 ± 0.4	20.7 ± 0.4
18:1n-7	1.8 ± 0.2	2.1 ± 0.2	12.5 ± 0.4	4.0 ± 1.1	4.2 ± 0.2
18:0	2.4 ± 0.2	19.9 ± 0.4	5.4 ± 0.2	3.0 ± 0.2	17.0 ± 0.3
20:4n-6, ARA	0.5 ± 0.1	5.1 ± 0.1	1.6 ± 0.0	0.7 ± 0.1	4.4 ± 0.1
20:5n-3, EPA	6.8 ± 0.3	2.9 ± 0.1	13.2 ± 0.3	8.1 ± 0.3	5.0 ± 0.1
20:2n-6/1n-11[2]	0.1 ± 0.0	0.3 ± 0.1	3.0 ± 0.0	0.7 ± 0.0	0.8 ± 0.1
20:1n-9/3n-3[2]	1.6 ± 0.3	0.2 ± 0.0	2.2 ± 0.2	1.7 ± 0.2	0.6 ± 0.0
20:1n-7	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:6n-3, DHA	20.2 ± 0.1	0.3 ± 0.0	14.1 ± 0.1	19.0 ± 0.1	3.1 ± 0.0
22:5n-3	0.4 ± 0.0	1.9 ± 0.0	1.1 ± 0.0	0.6 ± 0.0	1.7 ± 0.0
Unidentified	2.1 ± 1.3	6.5 ± 1.3	7.1 ± 0.2	3.1 ± 1.3	6.8 ± 2.8
Other fatty acids[3]	2.0	17.0	10.5	3.7	15.7
EPA/ARA	14.9 ± 1.4	0.6 ± 0.0	8.2 ± 0.1	11.6 ± 1.1	1.1 ± 0.0
DHA/EPA	3.0 ± 0.1	0.1 ± 0.0	1.1 ± 0.0	2.3 ± 0.1	0.6 ± 0.0
SFA	22.1 ± 0.2	39.7 ± 0.8	33.7 ± 1.3	24.5 ± 0.2	38.5 ± 0.7
MUFA	5.4 ± 0.8	34.3 ± 0.5	37.1 ± 0.3	11.7 ± 0.1	34.8 ± 0.4
PUFA	28.5 ± 0.8	26.6 ± 0.5	38.2 ± 0.4	30.5 ± 0.8	28.9 ± 1.2
Total fatty acids	58.1 ± 1.1	107.1 ± 1.4	116.1 ± 0.2	69.8 ± 2.6	109.0 ± 2.8

[1] minor lipid components present at < 1.0 mg g^{-1} dw include diacylglycerides and wax ester.
[2]Using HP-5 column these components coeluted; GC-MS analysis demonstrated 18:1n-9c, 20:2n-6 and 20:1n-9 to be the predominant components.
[3]fatty acids present in amounts < 1.0 mg g^{-1} dw included: 15:0, i15:0, 16:2n-7, 16:1n-5, i17:1, a17:1, 17:0, 18:3n-6, 18:1n-5, 18:4n- 3, i19:0; 20:2n-6, 20:3n-6, 20:4n-3, 20:1n-7, 20:0, 21:5n-3, 21:0, 22:4n-6, 22:5n-6, 22:4n-3, 22:1n-7 and 22:0.

6.5.2. Broodstock physiological changes

From pre- to post-starvation, the organ lipid index declined in both the digestive gland and the ovary of broodstock fed Diet SP and BP (Fig. 6.1). Recovery to pre-starvation concentrations in the digestive gland was attained within 1 month of re-feeding. In animals fed Diet SP, the digestive gland lipid index had a 15% reduction at both 3 and 5 months and increased by 41 % after 5 months in animals fed Diet BP. During the feeding treatments, the ovary lipid index only reached (fed Diet BP) or exceeded (fed Diet SP) pre-starved levels after 5 months.

The stage of ovarian development was predominantly Stage IV up to 3 months feeding with Stage V dominating the 5th month sampling (Table 6.2). Only 1 Stage VI ovary (final maturation stage) was noted during the 5th and final month of sampling. The gonadal somatic index (GSI) declined from pre- to post-starvation, remaining unchanged during feeding before doubling at the 5th month in animals from both dietary treatments (Fig. 6.2). Of the females that remained available for mating, 6 of 12 for Diet SP and 9 of 12 for Diet SP became ovigerous. These ovigerous females and 6 wild caught broodstock were used to monitor relative viable fecundity. There were no significant differences between the levels of fecundity from Diet SP, BP or wild broodstock with values of 534 ± 31, 677 ± 166 and 786 ± 145 (i.e., number of phyllosoma per mm CL of broodstock), respectively.

Table 6.2 The ovarian stage of development of *Jasus edwardsii* broodstock, determined macroscopically according to Fielder (1964), during periodic sampling.

Sampling time	Number of animals at ovarian stage			
	III	IV	V	VI
Pre-starvation (n=3)	1	2	-	-
Post-starvation (n=3)	-	3	-	-
1 month feeding (n=6)	-	6	-	-
3 month feeding (n=6)	-	4	2	-
5 month feeding (n=6)	-	-	5	1

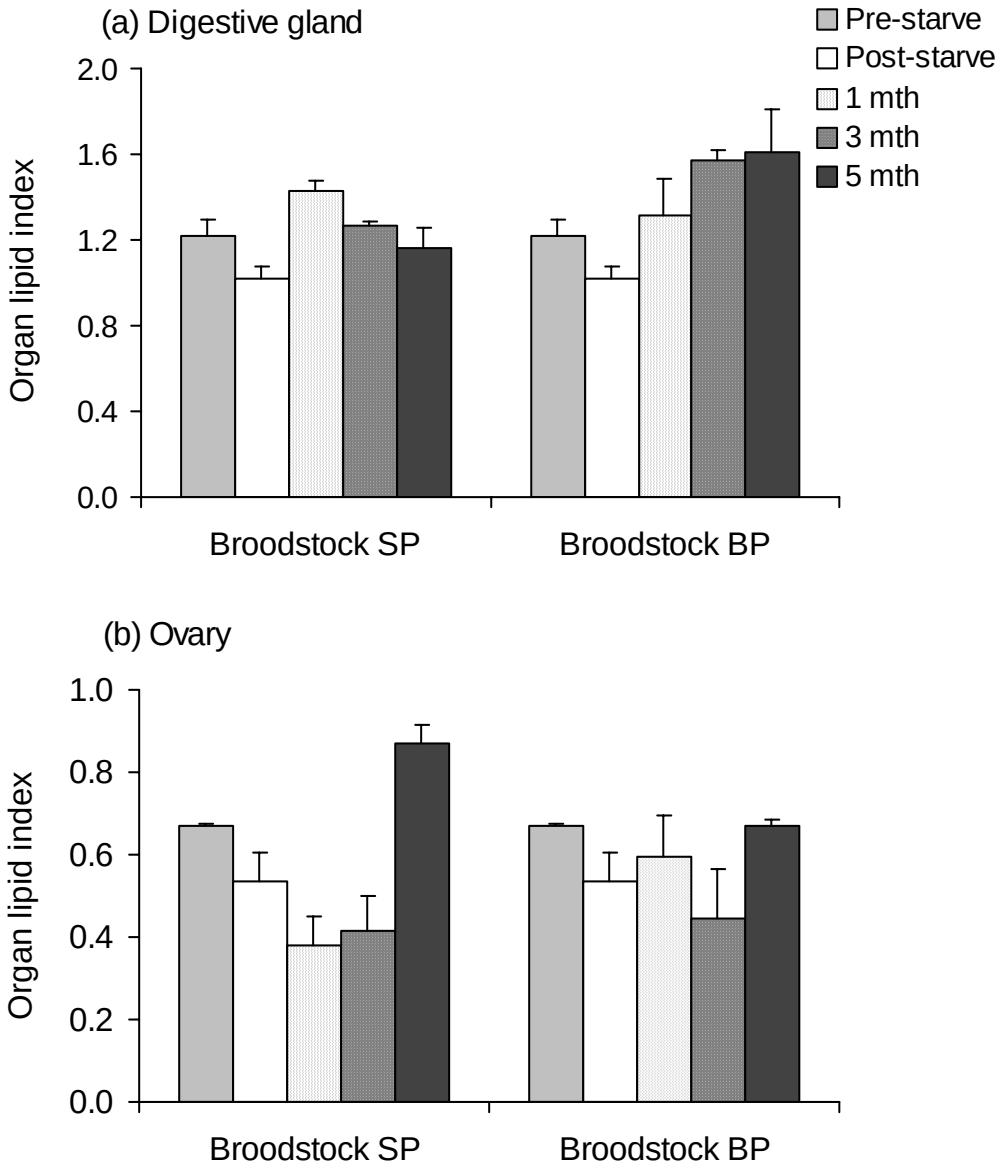

Fig. 6.1 The change in the organ lipid index (organ lipid weight/total body weight x 100) of female *Jasus edwardsii* fed either Diet SP (Broodstock SP) or Diet BP (Broodstock BP) for 5 months. Diets SP and Diet BP consisted of squid: pellet, 4:1 or beefheart: pellet, 4:1, respectively. Data are presented as mean ± sem (n=3).

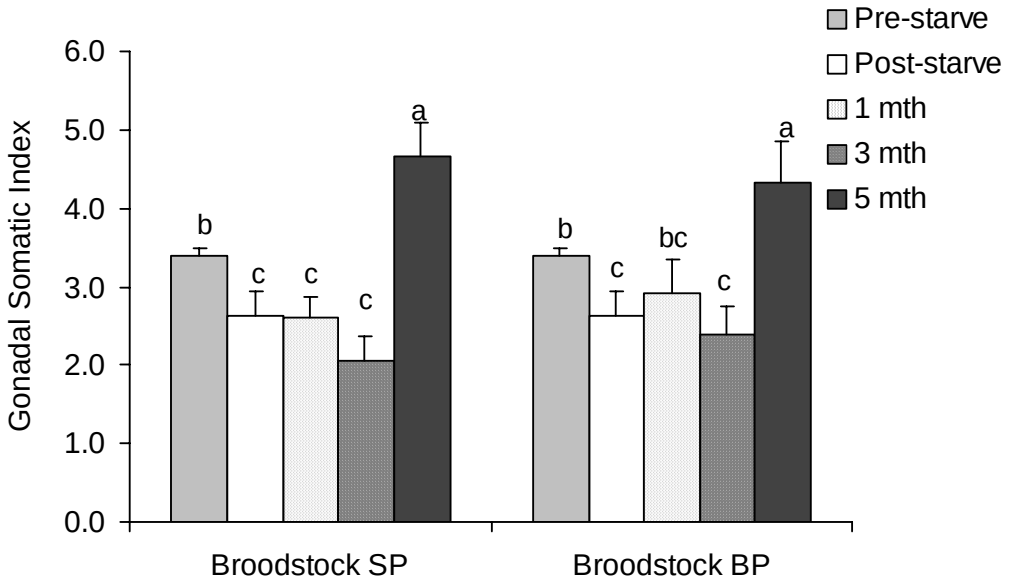

Fig. 6.2 The change in the gonadal somatic index (GSI, ovary weight/total body weight x 100) of female *Jasus edwardsii* broodstock after feeding for 5 months. Animals were fed either Diet SP (Broodstock SP) or Diet BP (Broodstock BP) (see Fig. 6.1 for details). Data are presented as mean ± sem (n=3). Different superscripts denote a significant difference between dietary phyllosoma groups within a moult stage.

6.5.3. Phyllosoma physiological characteristics

The size of eggs or phyllosoma to Stage IV did not differ between treatments but, at Stage IV, wild phyllosoma (from wild ovigerous females) were significantly larger than, phyllosoma SP (from broodstock fed Diet SP) or phyllosoma BP (from broodstock fed Diet BP) (Fig. 6.3). For all treatments, phyllosoma survival declined to Stage IV. However, survival was significantly greater for wild phyllosoma, by Stage IV they achieved 76% higher survival compared to phyllosoma from the best surviving broodstock dietary treatment (phyllosoma SP) (Fig. 6.4).

For phyllosoma submitted to the activity test, there was a significant interaction between temperature, salinity and phyllosoma activity (Fig. 6.5). The 9 combinations of temperature (21, 23 and 25ºC) and salinity (8, 10 12‰) produced a wide range of stress indices, with wild phyllosoma generally producing the lower stress indices compared to phyllosoma SP and BP.

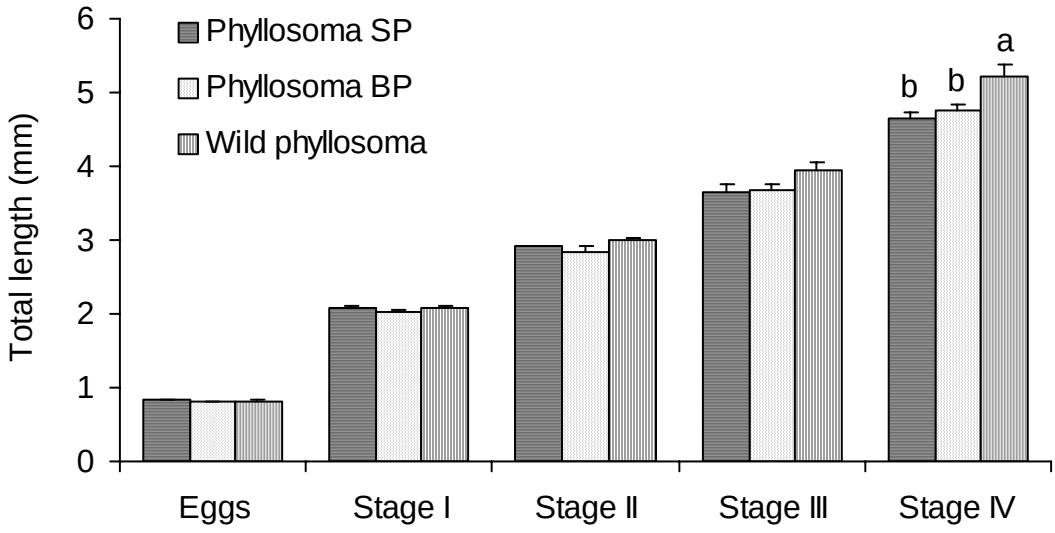

Fig. 6.3 Cross-sectional diameter of eggs and total length of Stages I to IV *Jasus edwardsii* phyllosoma from different broodstock dietary regimes and Wild Phyllosoma (see Fig. 6.1 for details). Data are presented as mean ± sem. For all samples n=3, except Phyllosoma SP and Wild Phyllosoma where n=6 and Phyllosoma BP where n=9. Different superscripts denote a significant difference between dietary phyllosoma groups within a moult stage.

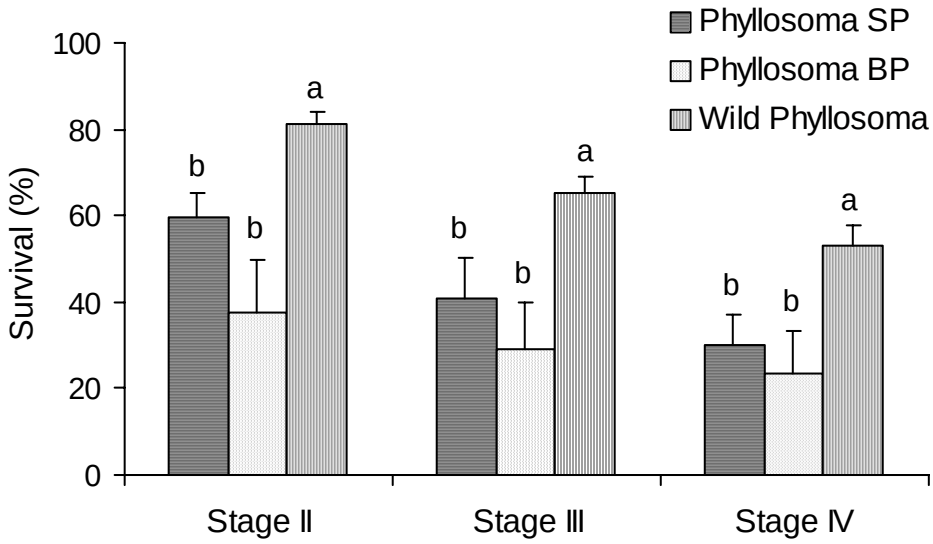

Fig. 6.4 Survival of *Jasus edwardsii* phyllosoma to Stage IV derived from broodstock fed Diets SP, BP and wild phyllosoma (see Fig. 6.1 for details). Data are presented as mean ± sem for Phyllosoma SP and Wild Phyllosoma where n=6 and Phyllosoma BP where n=9. Different superscripts denote a significant difference between dietary phyllosoma groups within a moult stage.

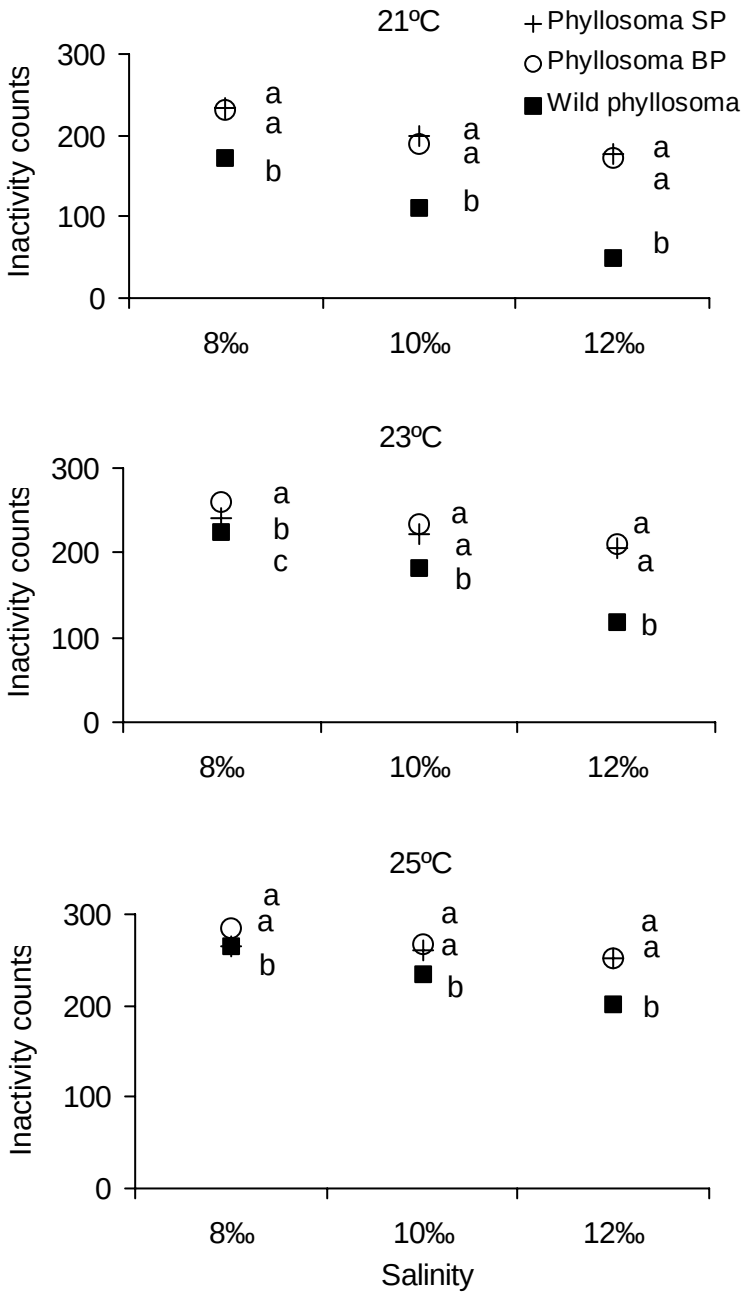

Fig. 6.5 Activity test measuring the number of newly-hatched *Jasus edwardsii* phyllosoma inactive and not responding to light stimuli at 3 min intervals for a total of 1 h ("stress index") when subject to different combinations of temperature (21, 23 and 25ºC) and salinity (8, 10 and 12‰). Phyllosoma resulted from broodstock fed different dietary regimes and reference phyllosoma (see Fig. 6.1 for details). Data are presented as mean ± sem for phyllosoma SP and wild phyllosoma where n=6 and phyllosoma BP where n=9. Different superscripts denote a significant difference between dietary phyllosoma groups at hatch.

There were several significant correlations between phyllosoma activity and survival in starvation LD-50's or culture to Stage IV (Table 6.3). The highest correlations were obtained with the activity parameters of 21ºC at 10‰. The equation of the linear fit between survival in culture and stress indices was: Survival = 71.84 – 0.3004 × stress index. A significant correlation was also present between phyllosoma LD-50 and survival to Stage IV (r = 0.9014).

Table 6.3 The correlation between activity levels of *Jasus edwardsii* phyllosoma subjected to different combinations of temperature and salinity against phyllosoma survival in an starvation LD-50 and animals cultured to Stage IV (42 d old fed phyllosoma).

Activity test		Starvation LD 50		Survival at Stage IV	
Temp (ºC)	Salinity	Correlation	Signif. Prob.	Correlation	Signif. Prob.
21	8	-0.67095	0.0121	-0.73963	0.0039
21	10	-0.80472	0.0009	-0.87597	0.0001
21	12	-0.79252	0.0012	-0.78547	0.0015
23	8	-0.52364	0.0663	-0.59314	0.0326
23	10	-0.55672	0.0481	-0.71027	0.0065
23	12	-0.60863	0.0273	-0.77041	0.0021
25	8	-0.35599	0.2325	-0.44185	0.1306
25	10	-0.49750	0.0836	-0.61513	0.0252
25	12	-0.62599	0.0221	-0.63446	0.0198

6.5.4. Broodstock lipid and fatty acid profiles

The digestive gland lipid dw was initially 6.8 g, this decreased to 5.8 g with starvation, rebuilding to 7.8 g and 10.9 g in animals fed Diet SP and BP, respectively (Table 6.4). Lipid contributed 71% to organ dw, was depleted by 7% during starvation and attained greater than pre-starvation lipid concentrations (73%) with feeding. The digestive gland lipid profile was dominated by TAG accounting for 90% of the total lipid dw prior and post starvation but declined to approximately 80% of the total lipid in the digestive gland at 5 months. Reductions in TAG were offset by increased PL in both dietary treatments.

Ovarian lipid inclusion declined from an initial weight of 3.7 g during 3 weeks starvation and even with refeeding to 3 months, was still ≤ 3.0 g. At 5 months lipid increased to 5.7 g and 4.6 g in animals fed Diets SP and BP, respectively and was associated with increased ovary weight of ≈ 300 % and ≈ 200 % in animals fed

Diets SP and BP, respectively. In spite of these significant changes in ovary lipid weights, the proportion of total lipid and lipid class compositions associated with the ovary remained relatively unchanged with diet or sampling time (Table 6.4). Lipid content was 40% of organ dw prior to starvation reducing to 38% after 5 months feeding, in spite of diet. The distribution of lipid classes consisted primarily of PL (50-54%), TAG (42-46%) and sterol (3-4%).

The content of lipid in the tail muscle remained relatively constant at 6 % of dw with both diet and time. PL was the dominant lipid class accounting for >90% of total lipid, peaking at between 93-95% at 3 months. Sterol was the only other lipid class present in excess of 1%.

The fatty acid profile of the digestive gland prior to the commencement of the study was dominated by a number of fatty acids including; 16:0, 18:1n-9, EPA and DHA (Fig. 6.6a, Table 6.5). Starvation had little or no effect on the qualitative (data not shown) distribution of fatty acids in the digestive gland. In animals fed Diet SP, the proportion of DHA gradually increased during the 5 month feeding period, while 18:2n-6 and 18:1n-9 decreased concomitant with dietary concentrations (Fig. 6.6a). Quantitatively, during the period following starvation and one month feeding on Diet SP, a 25% decrease in the content of total fatty acids resulted in reductions of a similar magnitude to individual fatty acids (Table 6.5). The most prominent reductions were evident in 16:0, 18:0, 18:1n-7, 18:1n-9, 18:2n-6 and EPA. After 5 months, only DHA attained a significantly greater content compared to before the commencement of the study, i.e. from 88.4 up to 139.8 mg g^{-1} dw.

Table 6.4 Lipid profiles and weight of *Jasus edwardsii* digestive gland, ovary and tail muscle at the commencement of the study (Pre-starve), after starvation for 3 weeks (Post-starve) and refeeding for 1, 3 and 5 months (just prior to the ovigerous moult). Animals were fed Diet SP (squid: pellet, 4:1) or Diet BP (beefheart: pellet, 4:1) to satiation. Data are presented as mean ± sem (n=3).

Composition (%)	Before feeding		Fed on Diet SP			Fed on Diet BP		
	Pre-starve	Post-starve	1 mth fed	3 mth fed	5 mth fed	1 mth fed	3 mth fed	5 mth fed
Digestive gland								
Triacylglycerol	89.6 ± 1.9	90.6 ± 2.8	87.4 ± 1.7	84.4 ± 0.7	80.9 ± 0.3	84.6 ± 2.9	82.3 ± 4.6	77.5 ± 2.8
Free fatty acid	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	1.3 ± 0.2	0.8 ± 0.0	0.2 ± 0.0	1.0 ± 0.2	0.9 ± 0.2
Sterol	0.4 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.9 ± 0.3	0.5 ± 0.1
Polar lipid	9.9 ± 2.2	9.2 ± 0.2	12.1 ± 0.1	13.6 ± 0.4	17.6 ± 0.0	14.9 ± 0.3	15.0 ± 6.0	21.1 ± 9.5
Organ lipid dw (g)	6.8 ± 0.1	5.6 ± 0.7	6.8 ± 0.1	7.7 ± 0.2	7.8 ± 2.0	6.7 ± 0.9	9.7 ± 0.2	10.9 ± 1.0
Organ dw (g)	9.6 ± 1.3	8.8 ± 1.1	9.9 ± 0.5	11.3 ± 0.4	10.1 ± 2.5	10.1 ± 1.2	14.5 ± 0.2	13.5 ± 2.6
Lipid % of organ dw	71.4 ± 2.3	64.4 ± 1.0	68.1 ± 0.4	68.0 ± 0.4	73.7 ± 0.7	66.6 ± 1.0	68.3 ± 0.8	73.6 ± 0.8
Ovary								
Triacylglyceride	43.4 ± 1.9	44.5 ± 1.0	45.9 ± 2.9	45.9 ± 0.2	42.4 ± 1.2	43.1 ± 1.2	45.2 ± 3.1	41.5 ± 1.0
Free fatty acid	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
Sterol	3.7 ± 0.4	3.6 ± 0.0	3.7 ± 0.4	3.1 ± 0.0	3.2 ± 0.2	3.0 ± 0.1	3.0 ± 0.8	3.8 ± 0.3
Polar lipid	52.4 ± 1.5	51.6 ± 1.1	50.1 ± 1.0	50.7 ± 1.6	54.0 ± 1.2	53.5 ± 2.9	51.4 ± 0.1	54.4 ± 0.5
Organ lipid dw (g)	3.7 ± 0.0	3.0 ± 0.5	2.1 ± 0.5	2.5 ± 0.5	5.7 ± 0.1	3.0 ± 0.6	2.7 ± 0.7	4.6 ± 0.2
Organ dw (g)	9.2 ± 0.1	7.0 ± 1.0	5.4 ± 0.8	5.5 ± 1.0	15.0 ± 0.9	7.2 ± 1.3	6.4 ± 1.3	12.1 ± 0.5
Lipid % of organ dw	40.7 ± 0.8	42.1 ± 0.6	39.2 ± 1.1	42.7 ± 0.2	38.1 ± 1.7	39.4 ± 1.4	41.8 ± 3.1	38.0 ± 1.2
Tail muscle								
Triacylglyceride	0.8 ± 0.6	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Free fatty acid	0.6 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
Sterol	7.6 ± 0.3	7.8 ± 0.0	4.8 ± 0.1	4.4 ± 0.3	5.8 ± 0.1	5.3 ± 0.6	4.2 ± 0.0	7.7 ± 0.2
Polar lipid	90.2 ± 0.3	91.4 ± 1.1	91.6 ± 3.6	94.6 ± 8.4	92.5 ± 2.4	92.6 ± 3.6	95.4 ± 2.3	91.7 ± 3.4
Lipid % of organ dw	6.8 ± 0.6	5.9 ± 0.1	5.2 ± 0.4	6.6 ± 0.4	5.9 ± 0.3	6.1 ± 0.7	7.6 ± 0.6	5.4 ± 0.4

Table 6.5 Quantitative fatty acid composition of female *Jasus edwardsii* digestive gland at the commencement of the study (Pre-starve), after starvation for 3 weeks (Post-starve) and refeeding for 1, 3 and 5 months (just prior to the ovigerous moult). Animals were fed Diet SP (squid: pellet, 4:1) or Diet BP (beefheart: pellet, 4:1) to satiation. Data are presented as mean ± sem (n=3).

Digestive gland	Before feeding		Fed on Diet SP			Fed on Diet BP		
Fatty acids mg g^{-1} dw	Pre-starve	Post- starve	1 mth	3 mth	5 mth	1 mth	3 mth	5 mth
14:0	16.1 ± 1.7	14.4 ± 1.0	11.9 ± 1.1	14.8 ± 0.8	16.2 ± 0.7	9.9 ± 1.6	12.4 ± 1.1	8.4 ± 0.4
16:1n-7	32.4 ± 1.4	29.3 ± 0.8	21.0 ± 3.8	23.7 ± 1.4	24.6 ± 0.3	16.7 ± 2.9	24.2 ± 0.3	18.5 ± 0.9
16:0	101.5 ± 7.3	98.0 ± 3.7	75.3 ± 9.6	102.6 ± 4.2	108.8 ± 8.0	85.4 ± 9.3	86.8 ± 5.7	77.7 ± 3.1
18:4n-3	7.2 ± 0.7	6.2 ± 0.3	4.1 ± 0.5	4.1 ± 0.1	3.7 ± 0.5	1.7 ± 0.3	2.3 ± 0.4	1.8 ± 0.2
18:2n-6	31.2 ± 2.6	32.5 ± 3.3	19.9 ± 0.4	21.8 ± 3.5	17.0 ± 2.2	22.2 ± 6.3	40.5 ± 3.6	49.3 ± 0.5
18:1n-9/3n-3[1]	80.9 ± 1.0	80.1 ± 6.7	57.4 ± 6.9	70.9 ± 5.2	66.2 ± 1.0	103.9 ± 16.0	100.4 ± 5.9	168.8 ± 18.4
18:1n-7	24.8 ± 0.9	22.5 ± 1.9	16.4 ± 1.7	21.9 ± 1.0	22.5 ± 1.5	16.9 ± 2.7	20.0 ± 0.7	22.3 ± 1.2
18:0	33.0 ± 2.0	36.7 ± 2.4	26.7 ± 3.0	34.3 ± 1.8	32.0 ± 0.5	59.8 ± 6.8	47.9 ± 3.8	66.9 ± 4.7
20:4n-6, ARA	8.6 ± 0.8	8.9 ± 0.5	5.8 ± 0.8	6.3 ± 0.4	6.2 ± 0.5	4.6 ± 1.5	11.0 ± 1.0	13.8 ± 0.3
20:5n-3, EPA	51.4 ± 3.5	48.7 ± 1.1	38.3 ± 2.8	39.2 ± 3.3	42.2 ± 1.6	7.3 ± 1.9	34.4 ± 1.6	18.9 ± 1.9
20:2n-6/1n-11[1]	8.3 ± 2.6	7.7 ± 0.9	6.8 ± 0.5	8.8 ± 1.3	10.6 ± 1.9	8.1 ± 2.8	4.3 ± 2.0	8.4 ± 1.0
20:1n-9/3n-3[1]	32.6 ± 3.4	28.6 ± 1.3	23.3 ± 1.9	38.9 ± 2.5	43.2 ± 2.7	9.4 ± 3.2	19.2 ± 6.3	13.6 ± 4.7
20:1n-7	6.0 ± 0.5	5.8 ± 0.8	4.3 ± 0.8	6.3 ± 0.3	6.0 ± 0.8	3.4 ± 0.7	4.8 ± 0.2	20.4 ± 10.1
22:6n-3, DHA	106.9 ± 9.5	96.4 ± 2.2	88.4 ± 7.8	105.3 ± 8.0	139.8 ± 5.0	9.6 ± 2.1	62.3 ± 3.7	32.8 ± 3.2
22:5n-3	9.7 ± 0.3	8.7 ± 0.8	7.0 ± 0.7	7.3 ± 0.4	8.3 ± 0.9	2.1 ± 0.7	7.8 ± 0.3	7.0 ± 0.2
Unidentified	42.5 ± 8.6	48.1 ± 4.5	27.7 ± 0.0	55.1 ± 1.7	63.9 ± 3.9	57.5 ± 6.4	46.2 ± 6.3	31.7 ± 4.7
Other[2]	34.7	32.6	30.4	25.2	28.7	37.3	45.2	59.9
EPA/ARA	6.0 ± 0.2	5.5 ± 0.2	6.7 ± 0.4	6.2 ± 0.2	6.9 ± 0.6	1.6 ± 0.2	3.1 ± 0.2	1.4 ± 0.2
DHA/EPA	2.1 ± 0.1	2.0 ± 0.0	2.3 ± 0.0	2.7 ± 0.0	3.3 ± 0.0	1.4 ± 0.1	1.8 ± 0.0	1.7 ± 0.0
SFA	168.7 ± 13.3	166.5 ± 4.9	125.7 ± 21.4	166.0 ± 5.2	174.3 ± 8.8	173.7 ± 17.2	164.1 ± 12.2	172.6 ± 7.8
MUFA	187.6 ± 5.0	173.9 ± 3.9	137.3 ± 5.7	169.7 ± 0.9	171.2 ± 3.0	168.5 ± 10.7	185.9 ± 3.1	266.2 ± 9.5
PUFA	229.0 ± 19.2	216.7 ± 8.1	174.0 ± 33.6	195.7 ± 15.5	230.5 ± 11.0	56.1 ± 28.8	173.5 ± 20.5	149.7 ± 4.8
Total	627.8 ± 38.2	605.2 ± 14.4	464.7 ± 46.1	586.5 ± 21.6	639.9 ± 23.7	455.8 ± 60.2	569.7 ± 31.7	620.2 ± 8.1

[1]Using HP-5 column in the GC, these components coeluted; GC-MS analysis demonstrated 18:1n-9c, 20:2n-6 and 20:1n-9 to be the predominant components.
[2]Other fatty acids present in amounts < 1.0 mg g-1 dw included: 15:0, i15:0, 16:2n-7, 16:1n-5, i17:1, a17:1, 17:0, 18:3n-6, 18:1n-5, i19:0; 20:3n-6, 20:4n-3, 20:0, 21:5n-3, 21:0, 22:4n-6, 22:5n-6, 22:4n-3, 22:0 and 22 NMIB.

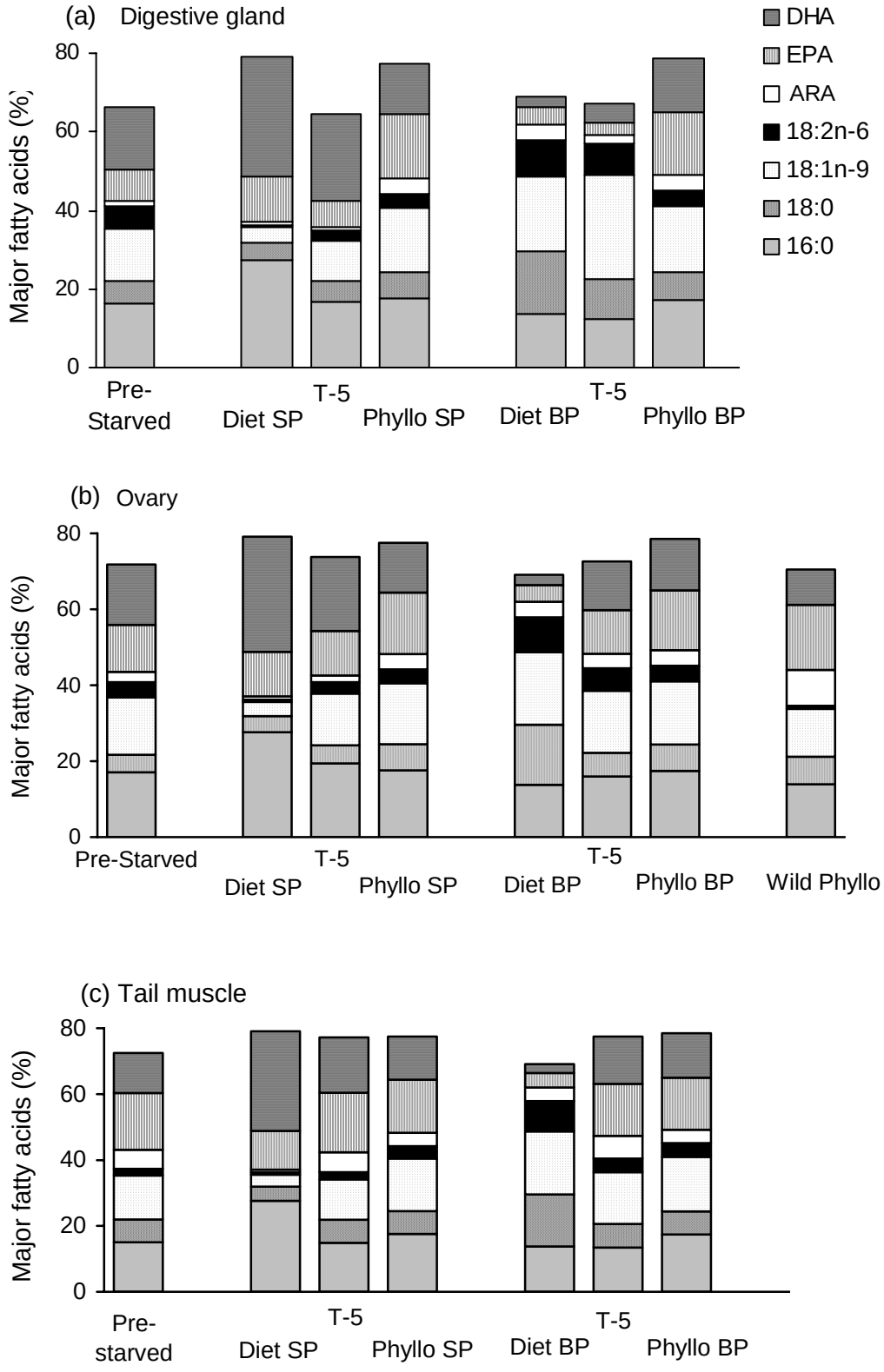

Fig. 6.6. The major fatty acids (%) in the (a) digestive gland, (b) ovary and (c) tail muscle of *Jasus edwardsii* broodstock prior to feeding (Pre-starve) and after feeding Diet SP or Diet BP (see Fig. 1 for details) for 5 months (T-5) . Diet and phyllosoma data are included for comparison. Phyllosoma resulting from broodstock fed Diet SP and BP are indicated by Phyllo SP and Phyllo BP, respectively. Wild Phyllosoma (Wild Phyllo) were sourced from wild-caught ovigerous females. For all samples n=3, except Phyllo SP and Wild Phyllo where n=6 and Phyllo BP where n=9.

In animals fed Diet BP, there were qualitative changes to the digestive gland composition. After 5 months feeding, the fatty acid profile of the digestive gland was similar to the diet, with increases to 18:0, 18:1n-9 and 18:2n-6 offset by reductions to EPA and DHA (Fig. 6.6a). In animals fed Diet BP, there was a quantitative decrease in total fatty acids after 1 month feeding with a gradual rebuilding of fatty acid content to pre-starvation values by 5 months. The C 18 series fatty acids, 18:0, 18:1n-9 and 18:2n-6, increased by approximately 50% while reductions of more than 60% occurred to EPA and DHA (Table 6.5). The digestive gland EFA ratios reflected the diet, with the ratio of EPA/ARA and DHA/EPA in Diet SP (11.6 and 2.3) producing elevated ratios in the digestive gland (6.9 and 3.3), while those receiving Diet BP (1.1 and 0.6) saw reduced ratios (1.4 and 1.7).

The qualitative fatty acid profile of the ovary did not change with starvation (data not shown) or with feeding Diet SP for 5 months (Fig. 6.6b). Feeding Diet BP for 5 months produced elevated concentrations of 18:2n-6 and ARA offset by reductions to 16:0 and DHA. During feeding of either diet, total ovary weight increased (Table 6.4) but fatty acid incorporation per gram of ovary declined (Table 6.6). This was particularly evident between the 3rd and 5th month for animals fed Diet SP where quantitative reductions in 18:2n–6, ARA and EPA occurred, while the incorporation of DHA remained stable. Animals fed Diet BP also demonstrated a quantitative reduction in incorporation to a range of fatty acids, the most prominent being 16:0, 16:1n-7, EPA and DHA while the content of 18:2n-6 and ARA increased. Changes to ovarian EFA ratios were concomitant with dietary concentrations; Diet SP demonstrated increased EPA/ARA (5.4 to 6.8) and DHA/EPA (1.4 to 1.7) ratios, while animals receiving Diet BP experienced a reduction in both the EPA/ARA (5.4 to 3.1) and DHA/EPA ratio (1.3 to 1.1). Correlations were not significant between individual ovarian fatty acids or fatty acid groups and relative viable fecundity.

Of the tissues examined, the tail muscle fatty acid profile was the least affected by broodstock dietary manipulation (Fig. 6.6c). Tail muscle was dominated by DHA, EPA, 18:1n-9, 18:0 and 16:0. Starvation caused qualitative reductions in EPA from 17.2 to 13.7%, and increases in ARA from 5.8 to 7.2% with all other

fatty acids relatively unaffected (data not shown). Feeding Diet SP for 5 months increased DHA concentration from 12.2 to 16.8%. Animals fed Diet BP also experienced increases to DHA, ARA, 18:1n-9 and 18:2n-6 (offset by reductions to 16:0, 18:1n-7 and EPA, Fig. 6.6c). Quantitatively, similar increases in individual fatty acids were evident (Table 6.7). In animals fed Diet SP, the EPA/ARA ratio increased from pre-starvation levels of 3.0 to 3.1 and the DHA/EPA ratio from 0.7 to 0.9, while animals receiving Diet BP demonstrated a reduction in the EPA/ARA ratio to 2.3 and increased their DHA/EPA ratio to 0.9, in contrast to the ratios in the diets.

Overall with the feeding of both diets, the EFAs ARA, EPA and DHA again reached pre-starvation levels or higher in the tail muscle and there were minor positive and negative changes in the ovary, while major changes were confined to the digestive gland.

Table 6.6 Quantitative fatty acid composition of female *Jasus edwardsii* ovary at the commencement of the study (Pre-starve), after starvation for 3 weeks (Post-starve) and refeeding for 1, 3 and 5 months (just prior to the ovigerous moult). Animals were fed Diet SP (squid: pellet, 4:1) or Diet BP (beefheart: pellet, 4:1) to satiation. Data are presented as mean ± sem (n=3).

Ovary	Before feeding		Fed on Diet SP			Fed on Diet BP		
Fatty acids mg g^{-1} dw	Pre-starve	Post-starve	1 mth	3 mth	5 mth	1 mth	3 mth	5 mth
14:0	8.1 ± 0.0	8.7 ± 0.4	9.2 ± 0.1	9.2 ± 0.7	8.4 ± 0.6	8.8 ± 0.1	7.3 ± 0.6	6.4 ± 1.1
16:1n-7	27.6 ± 1.5	28.7 ± 0.7	25.7 ± 2.7	24.7 ± 0.9	21.8 ± 2.3	25.1 ± 0.2	20.5 ± 1.6	20.1 ± 2.5
16:0	64.8 ± 0.8	65.1 ± 7.8	66.3 ± 4.0	61.5 ± 3.3	63.6 ± 2.6	63.0 ± 0.1	50.8 ± 1.6	51.0 ± 2.4
18:4n-3	3.1 ± 0.1	3.0 ± 0.1	2.8 ± 0.1	2.6 ± 0.2	2.0 ± 0.3	2.6 ± 0.1	2.0 ± 0.1	2.1 ± 0.2
18:2n-6	14.4 ± 1.1	15.4 ± 2.4	14.7 ± 1.0	13.9 ± 2.5	9.9 ± 0.6	18.2 ± 0.9	19.7 ± 1.3	19.2 ± 1.5
18:1n-9/3n-3[1]	54.2 ± 2.5	57.2 ± 6.5	58.8 ± 3.0	51.1 ± 3.7	44.8 ± 4.1	54.5 ± 0.1	52.4 ± 3.1	53.0 ± 8.4
18:1n-7	10.5 ± 0.2	9.9 ± 0.4	10.2 ± 0.2	8.9 ± 0.5	8.3 ± 0.7	10.2 ± 0.6	8.5 ± 0.3	7.9 ± 0.7
18:0	17.6 ± 1.3	17.7 ± 4.2	20.4 ± 0.4	15.6 ± 0.6	15.9 ± 1.0	19.7 ± 0.6	15.8 ± 2.2	19.5 ± 2.1
20:4n-6, ARA	8.1 ± 0.3	10.0 ± 0.4	8.2 ± 0.7	7.4 ± 0.5	5.9 ± 0.9	7.9 ± 0.8	10.7 ± 0.3	12.2 ± 1.2
20:5n-3, EPA	44.7 ± 2.9	46.4 ± 1.0	43.6 ± 1.2	42.3 ± 3.4	38.6 ± 2.7	42.2 ± 1.0	37.1 ± 1.0	36.4 ± 0.8
20:2n-6/1n-11[1]	2.9 ± 0.2	3.0 ± 0.1	3.7 ± 0.8	3.6 ± 0.8	3.1 ± 0.5	5.0 ± 0.5	4.6 ± 0.1	3.4 ± 0.1
20:1n-9/3n-3[1]	7.8 ± 0.9	5.0 ± 0.8	4.5 ± 2.6	7.2 ± 0.3	5.6 ± 2.2	6.6 ± 0.9	5.7 ± 0.7	5.1 ± 0.4
20:1n-7	1.3 ± 0.2	1.3 ± 0.0	1.2 ± 0.1	1.1 ± 0.0	3.4 ± 2.5	1.3 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
22:6n-3, DHA	61.6 ± 3.4	59.8 ± 2.3	61.8 ± 3.1	65.5 ± 2.4	64.1 ± 3.9	56.3 ± 2.1	52.1 ± 2.6	40.8 ± 2.6
22:5n-3	5.2 ± 0.4	4.7 ± 0.1	4.6 ± 0.5	4.7 ± 0.4	3.6 ± 0.5	4.8 ± 0.3	3.2 ± 0.1	3.7 ± 0.9
Unidentified	18.4 ± 0.2	19.8 ± 2.8	19.7 ± 4.5	16.8 ± 0.5	17.1 ± 2.0	18.9 ± 4.6	22.2 ± 1.2	22.0 ± 2.9
Other[2]	18.4	22.4	17.0	15.2	13.1	14.6	15.6	16.8
EPA/ARA	5.5 ± 0.0	4.7 ± 0.1	5.4 ± 0.3	5.7 ± 0.1	6.8 ± 0.7	5.4 ± 0.2	3.5 ± 0.2	3.1 ± 0.4
DHA/EPA	1.4 ± 0.0	1.3 ± 0.0	1.4 ± 0.0	1.6 ± 0.1	1.7 ± 0.1	1.3 ± 0.2	1.4 ± 0.0	1.1 ± 0.1
SFA	98.7 ± 2.4	100.7 ± 12.2	104.0 ± 7.2	94.4 ± 4.6	95.5 ± 3.3	99.7 ± 0.9	81.7 ± 3.3	86.7 ± 2.6
MUFA	103.1 ± 0.8	112.4 ± 3.0	103.4 ± 1.5	94.2 ± 0.9	88.6 ± 3.3	103.9 ± 1.9	94.4 ± 0.5	91.6 ± 4.1
PUFA	148.5 ± 4.0	145.2 ± 10.2	145.3 ± 16.0	145.9 ± 12.7	128.0 ± 12.2	137.2 ± 2.2	130.7 ± 7.2	120.1 ± 11.4
Total	368.7 ± 3.8	378.1 ± 26.0	372.4 ± 16.0	351.3 ± 19.3	329.2 ± 18.8	359.7 ± 12.5	329.0 ± 10.1	320.4 ± 19.1

See Table 6.5 for details.

Table 6.7 Quantitative fatty acid composition of female *Jasus edwardsii* tail muscle at the commencement of the study (Pre-starve), after starvation for 3 weeks (Post-starve) and refeeding for 1, 3 and 5 months (just prior to the ovigerous moult). Animals were fed Diet SP (squid: pellet, 4:1) or Diet BP (beefheart: pellet, 4:1) to satiation. Data are presented as mean ± sem (n=3).

Tail muscle	Before feeding		Fed on Diet SP			Fed on Diet BP		
Fatty acids mg g^{-1} dw	Pre-starve	Post-starve	1 mth	3 mth	5 mth	1 mth	3 mth	5 mth
14:0	0.4 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.8 ± 0.2	0.4 ± 0.0	0.4 ± 0.0
16:1n-7	1.8 ± 0.1	1.5 ± 0.1	1.2 ± 0.1	2.1 ± 0.3	2.0 ± 0.1	1.6 ± 0.0	1.9 ± 0.2	1.8 ± 0.1
16:0	6.3 ± 0.6	5.7 ± 0.2	5.2 ± 0.5	7.3 ± 0.6	6.2 ± 0.2	6.9 ± 0.2	6.8 ± 1.1	6.2 ± 0.4
18:4n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:2n-6	0.8 ± 0.1	0.9 ± 0.0	1.0 ± 0.4	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.2	2.2 ± 0.6	1.9 ± 0.1
18:1n-9/3n-3[1]	5.5 ± 0.5	4.7 ± 0.1	3.8 ± 0.3	6.5 ± 0.5	5.1 ± 0.1	6.1 ± 0.7	7.6 ± 1.5	7.2 ± 0.2
18:1n-7	1.9 ± 0.3	1.9 ± 0.1	1.6 ± 0.1	1.4 ± 0.2	1.0 ± 0.1	2.7 ± 0.8	1.1 ± 0.2	1.0 ± 0.1
18:0	2.9 ± 0.3	3.2 ± 0.4	2.6 ± 0.6	3.8 ± 0.4	2.9 ± 0.1	4.9 ± 1.6	3.9 ± 0.6	3.3 ± 0.1
20:4n-6, ARA	2.5 ± 0.3	2.7 ± 0.1	1.4 ± 0.1	2.6 ± 0.2	2.5 ± 0.4	3.6 ± 0.7	3.4 ± 0.4	3.1 ± 0.2
20:5n-3, EPA	7.3 ± 0.8	5.2 ± 0.2	4.1 ± 0.4	7.6 ± 1.0	7.5 ± 0.2	5.9 ± 0.6	7.7 ± 1.1	7.2 ± 0.2
20:2n-6/1n-11[1]	0.7 ± 0.1	1.2 ± 0.3	0.8 ± 0.3	0.7 ± 0.1	0.4 ± 0.1	1.3 ± 0.5	0.6 ± 0.1	0.5 ± 0.0
20:1n-9/3n-3[1]	0.5 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.0
20:1n-7	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:6n-3, DHA	5.1 ± 0.4	4.4 ± 0.2	4.2 ± 0.6	7.3 ± 0.9	7.0 ± 0.1	5.0 ± 0.2	6.9 ± 0.9	6.6 ± 0.1
22:5n-3	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.0	0.4 ± 0.0
Unidentified	3.4 ± 1.2	3.5 ± 0.0	3.0 ± 0.7	2.0 ± 0.5	2.0 ± 0.1	2.3 ± 0.1	4.1 ± 0.1	3.5 ± 0.2
Other[2]	2.3	1.7	1.5	2.5	2.3	2.8	1.4	1.9
EPA/ARA	3.0 ± 0.2	1.9 ± 0.1	3.0 ± 0.2	2.9 ± 0.2	3.1 ± 0.4	1.7 ± 0.1	2.3 ± 0.6	2.3 ± 0.2
DHA/EPA	0.7 ± 0.0	0.8 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.8 ± 0.0	0.9 ± 0.0	0.9 ± 0.0
SFA	11.6 ± 1.0	11.2 ± 0.7	9.6 ± 1.1	13.4 ± 1.2	11.2 ± 0.1	14.6 ± 2.2	12.8 ± 0.7	10.6 ± 0.2
MUFA	10.0 ± 0.4	8.9 ± 0.2	7.6 ± 0.6	11.1 ± 0.9	8.8 ± 0.0	12.1 ± 0.6	12.5 ± 0.3	11.6 ± 0.2
PUFA	17.0 ± 2.1	14.6 ± 0.5	11.6 ± 1.6	20.5 ± 2.9	19.5 ± 0.5	17.1 ± 3.7	19.9 ± 2.1	19.6 ± 0.6
Total	42.0 ± 3.9	38.2 ± 1.6	31.8 ± 2.7	47.0 ± 4.8	41.5 ± 0.5	46.1 ± 2.0	49.3 ± 6.5	45.8 ± 1.2

See Table 6.5 for details.

6.5.5. Phyllosoma lipid and fatty acid profiles

There was no significant difference between the qualitative lipid class profiles of phyllosoma SP, BP or wild phyllosoma groups (Table 6.8). PL was the dominant lipid class (86 – 90%) with sterol of secondary importance (9-11%), while TAG and FFA were minor lipid components each contributing < 2%. When expressed quantitatively, phyllosoma BP had approximately 50% more total lipid, PL and sterol than phyllosoma SP or wild phyllosoma.

The qualitative fatty acid profile of phyllosoma from dietary treatments did not differ from each other but both differed from wild phyllosoma (Fig. 6.6, Table 6.8). Phyllosoma from dietary treatments contained elevated concentrations of 16:0, 18:2n-6, 18:1n-9 and DHA with lower concentrations of 17:0, 18:1n-7 and ARA compared to wild phyllosoma. The balance of fatty acids present in phyllosoma SP and BP did not reflect broodstock dietary inclusion (Fig. 6.6b).

Quantitatively, the only difference between phyllosoma from the two dietary treatments was minor; phyllosoma BP contained more DHA (Table 6.8), in spite of this broodstock treatment being fed the lowest dietary ration of DHA (Table 6.1), resulting in ovaries with the lowest concentration of the EFA. DHA and EPA were present at a higher concentration in phyllosoma from the broodstock dietary treatments compared to wild phyllosoma, while ARA was less abundant, resulting in EPA/ARA ratios 2-3 times larger than found in wild phyllosoma. The pattern of fatty acid abundance reflected the qualitative data (Table 6.8) while the total saturated fatty acids (SFA), MUFA and PUFA and the total fatty acids in phyllosoma BP were > 1.5 times the content of wild phyllosoma.

Table 6.8 Qualitative and quantitative lipid and fatty acid profiles of newly-hatched phyllosoma from broodstock either fed Diet SP consisting of squid: pellet (4:1) (Phyllo SP) or Diet BP consisting of beefheart: pellet (4:1) (Phyllo BP), or from wild phyllosoma (Wild Phyllo) sourced from wild ovigerous females. Data are presented as mean ± sem (n=3).

	Qualitative data (% fatty acid)			Quantitative data (mg g^{-1} dw)		
	Phyllo SP	Phyllo BP	Wild Phyllo	Phyllo SP	Phyllo BP	Wild Phyllo
Lipids						
Triacylglyceride	0.9 ± 0.3	0.5 ± 0.4	0.6 ± 0.4	1.2 ± 0.4	0.9 ± 0.3	0.7 ± 0.4
Free fatty acid	0.6 ± 0.2	1.8 ± 1.1	0.6 ± 0.2	0.6 ± 0.2 [b]	3.3 ± 0.9 [a]	0.7 ± 0.2 [b]
Sterol	11.1 ± 1.1	10.7 ± 0.6	8.7 ± 0.5	14.3 ± 0.4 [b]	19.4 ± 0.7 [a]	10.1 ± 1.1 [b]
Polar lipid	86.2 ± 0.6	86.2 ± 1.8	89.6 ± 1.0	110.1 ± 0.0 [b]	155.8 ± 2.9 [a]	104.2 ± 11.0 [b]
Other lipids[1]	1.2 ± 0.2	0.8 ± 0.1	0.5 ± 0.4	1.0 ± 0.4	1.4 ± 0.6	0.5 ± 0.4
Total lipid content				127.2 ± 3.6 [b]	180.8 ± 4.9 [a]	116.2 ± 12.0 [b]
Fatty acids						
14:0	0.8 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
16:1n-7	3.3 ± 0.3	2.7 ± 0.5	3.2 ± 0.3	3.7 ± 0.5	3.3 ± 0.7	2.2 ± 0.2
16:0	17.6 ± 0.4 [a]	17.4 ± 0.2 [a]	13.9 ± 0.0 [b]	19.7 ± 2.2 [a]	20.8 ± 1.1 [a]	9.7 ± 1.0 [b]
17:0	0.8 ± 0.0 [b]	0.7 ± 0.0 [b]	1.5 ± 0.0 [a]	0.9 ± 0.1	0.9 ± 0.0	1.0 ± 0.1
18:2n-6	3.7 ± 0.3 [a]	4.1 ± 0.1 [a]	0.8 ± 0.1 [b]	4.0 ± 0.2 [a]	4.9 ± 0.3 [a]	0.6 ± 0.1 [b]
18:1n-9/3n-3[2]	16.0 ± 0.1 [a]	16.6 ± 0.7 [a]	12.6 ± 0.1 [b]	18.0 ± 1.9 [a]	19.9 ± 1.5 [a]	8.8 ± 0.3 [c]
18:1n-7	3.4 ± 0.2 [b]	3.2 ± 0.1 [b]	5.5 ± 0.6 [a]	3.9 ± 0.6	3.9 ± 0.2	3.8 ± 0.3
18:0	6.9 ± 0.1	7.0 ± 0.2	7.3 ± 0.1	7.7 ± 0.8 [a]	8.4 ± 0.5 [a]	5.1 ± 0.5 [b]
20:4n-6, ARA	4.0 ± 0.7 [b]	4.1 ± 0.3 [b]	9.4 ± 0.1 [a]	4.5 ± 0.7 [b]	4.9 ± 0.5 [b]	6.6 ± 0.3 [a]
20:5n-3, EPA	16.2 ± 0.4	15.8 ± 0.3	17.2 ± 0.5	18.0 ± 1.6 [a]	18.8 ± 0.7 [a]	11.8 ± 0.8 [b]
20:2n-6/1n-11[2]	2.1 ± 0.0	2.1 ± 0.1	1.6 ± 0.1	2.3 ± 0.2	2.5 ± 0.1	1.2 ± 0.2
20:1n-9/3n-3[2]	2.3 ± 0.3	2.2 ± 0.1	3.0 ± 0.1	2.6 ± 0.5	2.6 ± 0.2	2.1 ± 0.2
22:6n-3, DHA	13.1 ± 0.8 [a]	13.5 ± 0.6 [a]	9.3 ± 0.2 [b]	14.7 ± 0.8 [b]	16.1 ± 0.3 [a]	6.5 ± 0.6 [c]
22:5n-3	0.7 ± 0.0	0.7 ± 0.0	1.6 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	1.1 ± 0.2
Unidentified	2.6 ± 0.2	2.6 ± 0.2	4.7 ± 0.5	3.0 ± 0.5	3.2 ± 0.4	2.5 ± 0.8
Other fatty acids[3]	6.5	7.3	7.6	7.1	7.3	6.0
EPA/ARA				4.3 ± 0.6 [ab]	4.0 ± 0.4 [b]	1.8 ± 0.1 [c]
DHA/EPA				0.8 ± 0.1 [a]	0.9 ± 0.0 [a]	0.5 ± 0.0 [b]
SFA	28.2 ± 0.4	28.0 ± 0.3	28.2 ± 0.4	31.6 ± 3.3 [a]	33.4 ± 1.7 [a]	19.6 ± 1.1 [b]
MUFA	26.7 ± 0.7	26.5 ± 0.5	27.8 ± 0.3	30.0 ± 3.7 [ab]	31.7 ± 1.9 [a]	19.3 ± 0.4 [c]
PUFA	41.2 ± 0.8	41.6 ± 0.3	43.8 ± 0.6	45.8 ± 3.8 [ab]	49.5 ± 2.1 [a]	30.6 ± 0.1 [c]
Total				111.9 ± 11.4 [a]	119.3 ± 6.0 [a]	69.6 ± 3.7 [b]

[1]minor lipid components present at < 1.0 mg g^{-1} dw include diacylglycerides and wax ester.
[2]Using HP-5 column in the GC these components coeluted, GC-MS analysis demonstrated 18:1n-9c, 20:2n – 6 and 20:1n – 9 to be the predominant components.
[3]Other fatty acids present in amounts < 1.0 mg g^{-1} dw included: 15:0, i15:0, 16:2n-7, 16:1n-5, i17:1, a17:1, 17:0, 18:3n-6, 18:1n-5, i19:0; 20:3n-6, 20:4n-3, 20:0, 21:5n-3, 21:0, 22:4n-6, 22:5n-6, 22:4n-3, 22:0 and 22 NMIB.

6.6. Discussion

6.6.1. Dietary influence on lipid storage and reproductive development

Growth and fecundity of an organism depends upon a number of environmental and physical factors, including the impacts of food quantity, composition and seasonal variability (Joshi and Diwan, 1996). An important nutritional component of food, which may vary widely, is lipid (Sargent, 1995). In *J. edwardsii*, total lipid increased with feed duration (digestive gland) and reproductive development (ovary). While the pattern of lipid class distribution remained constant and typical of that found in other crustaceans (Alava et al., 1993). TAG (energy source) dominated the digestive gland, muscle contained primarily PL (maintenance of membrane structures) and the ovary had similarly large proportions of PL and TAG (egg production requiring both membranes and energy).

In crustaceans, the digestive gland processes and stores lipids (Vogt et al., 1985). Storage products may then be mobilized for specific purposes including ovarian maturation, ecdysis or metabolism (O'Connor and Gilbert, 1968; Lawrence, 1976; Reid and Caulton, 1980). In this study we found that adult *J. edwardsii* accumulated a high concentration of lipid in the digestive gland (66-74% dw), in excess of juveniles (≤ 41% dw) (Johnson et al., 2003; Ward et al., 2003) and many other crustaceans e.g., 5-14% in *Penaeus japonicus* (Teshima et al.,1989) and 11% in *P. indicus* (Cahu et al.,1995), which suggests that lipid is stored for a specific purpose. In this instance, there was no apparent reduction to digestive gland lipid stores associated with ovarian maturation as expected in crustaceans (Adiyodi and Adiyodi, 1971; Allen, 1972; Castille and Lawrence, 1989). However, the period following the pre-ovigerous molt (≈ 3 weeks prior to mating and egg extrusion) was not sampled and therefore the use of digestive gland lipid stores during this stage cannot be ruled out. Maternal food intake ensured that digestive gland lipid stores were maintained or even increased despite increased ovarian lipid content during maturation. The use of dietary supply rather than digestive gland reserves to supplement ovarian requirements is not a common strategy. However, a similar

mechanism has been reported by Clark (1982) in polar shrimp and indicates that nutrient supply during maturation is adequate to meet requirements.

There appeared to be minimal lipid mobilization from the muscle to the ovary, which agrees with findings in penaeids (Lawrence et al., 1979). Even though there was a decrease in the ovarian lipid content and dw during starvation, and a significant increase with ovarian maturation the fatty acid profile of the ovary, like the tail muscle, showed little change and was independent of diet.

6.6.2. Broodstock lipid and fatty acid storage and use

If digestive gland lipid deposits have only minimal involvement in ovarian maturation this poses the question of the purpose for such a large lipid store. The answer is likely to lie in the seasonal fluctuations in the quality and quantity of food in their natural habitat (Jernakoff et al., 1993; Barkai et al., 1996). Having such a large energy store would be a useful environmental adaptation to circumvent periods of inadequate nutrition (Lawrence, 1976), particularly as the majority of the lipid in the digestive gland is present as triacylglycerol, the most common source of lipid associated with energy metabolism (Sargent, 1995). Supporting this theory is the reduction to digestive gland lipid and in particular, total fatty acids that occurred during starvation and 1 month post-starvation as animals adjusted to a new food source. We speculate that the increase in digestive gland lipid stores over that previously evident in juveniles (Johnson et al., 2003; Ward et al., 2003) may indicate a shift in the ability of animals to utilize lipid as an energy source with adulthood. The use of lipids and fatty acids as energy sources occurs in other stages of development in *J. edwardsii*. In the period following metamorphosis from phyllosoma to puerulus, lipid stores are heavily sequestered to sustain metabolism during a prolonged non-feeding period prior to settlement (Jeffs et al., 2001).

One of the most interesting outcomes of the study was how the various organs and tail muscle responded to differing lipid and fatty acid dietary profiles. Ovarian and tail muscle lipid and fatty acid profiles were maintained across treatments in spite of dietary differences. This was in contrast to the plasticity of the digestive gland fatty acid profile, which attained that of the dietary source, and reinforces the suggestion that the digestive gland is the major lipid and fatty acid storage site for

crustaceans (Vogt et al., 1985; Harrison, 1990; Cahu et al., 1994). In their natural habitat spiny lobsters are selective foragers (Fielder, 1965; Barkai et al., 1996). The combination of this trait and the pliant nature of digestive gland may prove a useful tool in identifying the dietary preferences that help maximize wild egg and larval quality.

6.6.3. Phyllosoma lipid and fatty acid profiles

The ability of crustacean broodstock to produce competent larvae often declines in captivity (Harrison, 1990; Palacios et al., 1998). While stress and environmental factors contribute to this decline, the ability of an animal to sequester adequate nutrient reserves prior to and during oocyte development can also be a major impediment (Teshima et al., 1983, 1989; Palacios et al., 1998). This may have been a concern if the lipid and fatty acid profile of the eggs and lecithotrophic larvae had reflected that of the broodstock diet, as is often the case (Cahu et al., 1986, 1994; Watanabe, 1995). Instead, a predefined lipid and fatty acid profile was maintained, largely in spite of dietary differences, while not common, has been noted previously (Cahu et al., 1995). This suggests that gross energy supply is an important factor in the nutrition of this species and substantiates a view that broodstock fed to excess, even with an inappropriate mix of EFA, will use a variety of mechanisms to produce viable larvae (J. Sargent, pers comm.). The sequestering of the required lipids and fatty acids required during broodstock maturation negated the ability of activity tests to differentiate between what may be relatively more important lipid components. In some species, the complete exclusion of HUFA from the broodstock diet for short periods can still produce viable larvae albeit at the expense of fecundity and egg hatchability (Wen et al., 2002). During this study, while the HUFA supply differed markedly between diets, it appeared adequate to have minimal effects upon relative viable fecundity.

Elevated levels of DHA were present in phyllosoma fed Diet BP, the lowest source of DHA, possibly due to an overcompensation mechanism maximizing the sequestering of essential reserves, since *de novo* synthesis of DHA by crustaceans is minimal (Kanazawa et al., 1979) or non-existent (Castell 1983; Harrison, 1990; Teshima et al. 1992). All fatty acids, with the exception of ARA, were present in

excess of that found in wild phyllosoma, no link could be established between fatty acid abundance and larval survival in culture.

6.6.4. Phyllosoma physiological parameters

A greater capacity to select competent larvae for culture was obtained by using the stress parameters of 21ºC at 10‰ compared to that previously defined (Smith et al. 2003b). Additionally, the benefit of a LD-50 as a predictive tool for larval competency, rather than being used to elucidate toxicity concentrations (Gerking and Lee, 1980; Sarada and Pillai, 1993), was noted. The results of the LD-50 were highly correlated with survival in culture however, its use for this purpose is questionable due to the long lead time required to obtain results, a period of 1-2 weeks, far in excess of the 1 hr duration of the activity test. The performance of larvae in activity tests have been associated with the larval content of specific fatty acids and in particular their EFA profile (Rees et al., 1994; Abi-ayad et al., 1995; Kanazawa, 1997). While the influence of fatty acids was not apparent in this study, it is considered that nutrition may have had an impact upon the functionality of activity tests. For example, ascorbic acid improves activity test results in crustaceans in association with an animal's ability to osmoregulate (Moreau et al., 1998).

A greater proportion of broodstock fed Diet BP extruded eggs compared to those fed Diet SP. This demonstrates that relatively inexpensive ingredients, such as beefheart, when used in conjunction with HUFA aquafeeds may reduce the reliance on expensive marine dietary items (squid) during the process of maturation in *J. edwardsii* broodstock. It should be noted though, that the concentrations of 22:6n-3 and to lesser extent 20:5n-3, decreased significantly by the 5th month of feeding Diet BP in digestive gland (affecting reserves), ovary (potentially affecting future reproductive events), but not in the tail muscle (maintenance of self the highest priority). Overall, wild phyllosoma were larger, had higher survival (at Stages II, III and IV), much lower levels of all major fatty acids (except 20:4n-6) and lower inactivity counts (stress index) and therefore a higher chance of survival to Stage IV, compared to phyllosoma fed either diet. The cause of these differences between wild and dietary phyllosoma needs to be established, as the results from the current

study suggests that high PL diets, either with or without large proportions of n-3 HUFA, could not elicit the same responses.

6.7. Conclusion

The digestive gland, ovary and tail muscle lipid class profiles in *J. edwardsii* were rigid, subject to minimum alteration via dietary input, as were the fatty acid profiles of ovarian and tail muscle tissue. This was in contrast to the plasticity of the fatty acid profile of the digestive gland. From an ecological viewpoint, this study demonstrates that broodstock are able to survive and produce viable larvae on a diet containing low concentrations of EFA and suggests a capacity for them to also do so in the wild. From an aquaculture perspective, the ability of animals to selectively sequester a range of lipid and fatty acids from fresh dietary components somewhat reduces the emphasis on providing exact amounts of particular fatty acids, and may broaden the nutritional emphasis to include other dietary factors. An improved activity test was developed to measure larval competency. It is probable to suggest that the activity test may also be used to monitor larval competency at later developmental stages. The use of digestive gland fatty acid profiles in combination with lipid signatures may be a useful diagnostic tool for elucidating natural dietary components.

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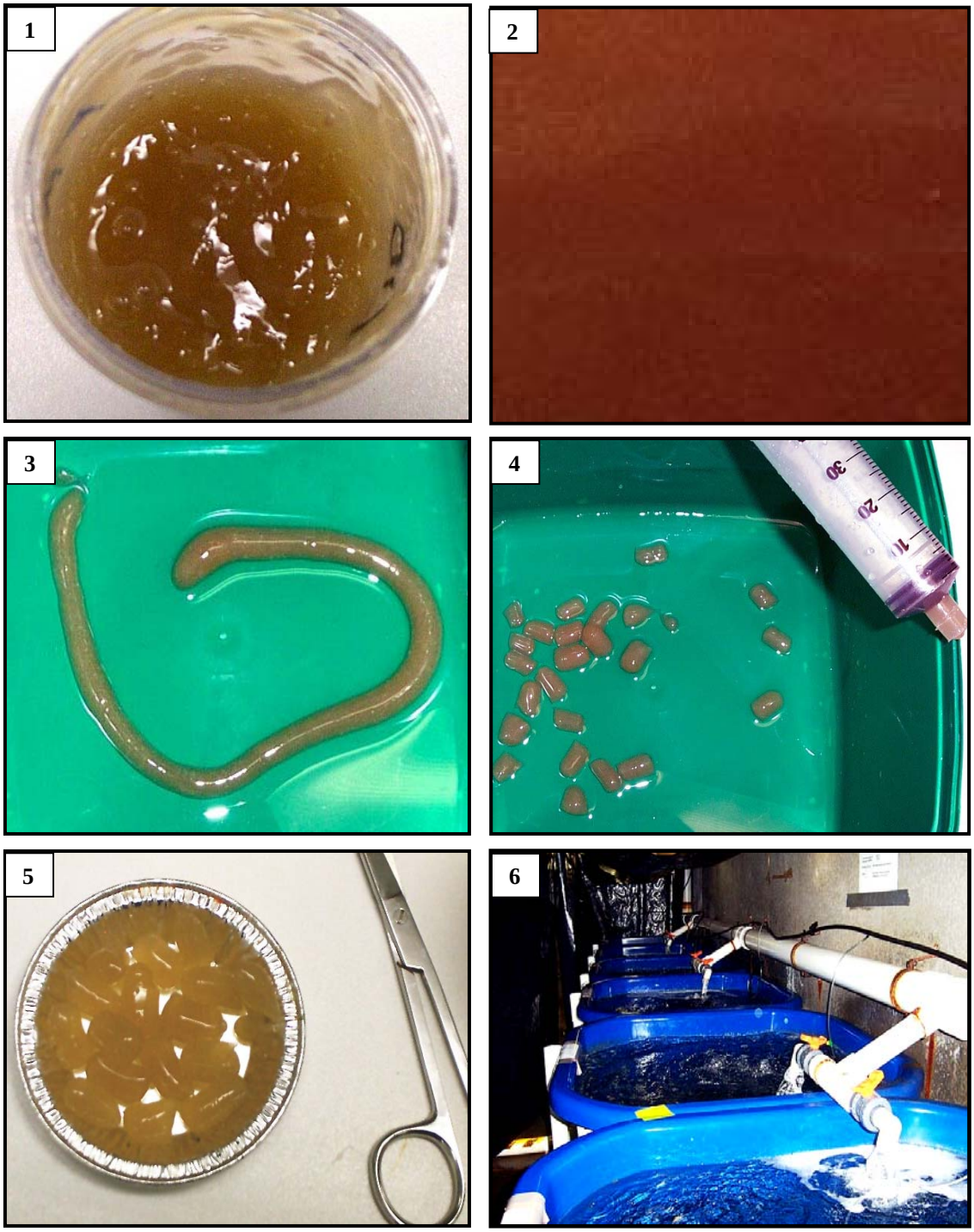

Plate 7. Manufacture of ascorbic acid pellets and the broodstock experimental system. (1) 12% Alginate. (2) *Artemia* biomass. (3) Alginate and *Artemia* mix (1:1) extruded through a 60 mm syringe into a 4% CaCl solution. (4) Extruded mix cut into 1 cm long pellets. (5) *Artemia* pellets prepared ready for use. (6) Broodstock tanks used during the 10-week broodstock ascorbic acid supplementation study.

Plate 7.

7.1. Abstract

A novel ascorbic acid (AA) supplement consisting of AA-enriched juvenile *Artemia* encapsulated within an alginate pellet was used to boost the AA content of *Jasus edwardsii* broodstock diets. The concentration of AA in *Artemia*-encapsulated pellets ranged from 150 μg g^{-1} when using unenriched *Artemia* to 9 153 μg g^{-1} with enriched *Artemia*. Four experimental diets were fed to broodstock and consisted of either a basal diet alone (AA concentration of 150 μg g^{-1}) containing mussels, squid and compound prawn diet, or the basal diet plus *Artemia*-encapsulated pellets. Graduated AA concentrations of 150 (low), 450 (med) or 1350 (high) μg g^{-1} were obtained by utilizing different combinations of unenriched and AA-enriched *Artemia* in combination with the basal diet. Dietary supplementation of med and high concentrations resulted in saturation of ovarian AA concentrations at ≈ 240 μg g^{-1}. Total ovarian AA content increased a modest 160%, proportionally much less than the 900% difference between low and high dietary supplementation. Concentrations of AA in the digestive gland of broodstock fed diets containing ≤ 450 μg AA g^{-1} were in the range of 76 to 92 μg AA g^{-1}, increased supplementation (1350 μg AA g^{-1}) demonstrated the storage capacity of this organ (270 μg AA g^{-1}). The tail muscle contained a significant store of AA, this was reduced in content in animals receiving little or no AA supplementation, concomitant increases in ovarian AA suggests it is a source of AA for the ovary during maturation. AA supplementation had no significant effect on relative viable fecundity (total number of phyllosoma produced adjusted for female size) although increasing AA supplementation reduced the variability of this parameter. The increase in dietary AA content resulted in a dose-dependent increase in AA content of eggs and phyllosoma. There was minimal AA depletion during embryonic development suggesting that AA is not utilized appreciably during this period and no effect of AA supplementation on phyllosoma quality, assessed by larval activity tests, size and survival in culture. The maximum endogenous increases in AA content of eggs and phyllosoma were modest (33%) and were a function of the ability to increase ovarian AA content, which had a saturation point already close to the content

provided by the basal diet. To elicit more pronounced increases in larval AA concentrations, it may be necessary to directly feed AA supplements to phyllosoma.

Key words: *Artemia*, ascorbic acid, *Jasus edwardsii*, larval quality, maturation diet.

7.2. Introduction

Problems of inconsistent egg and larval quality exist with the culture of the spiny lobster, *Jasus edwardsii*. Other crustaceans demonstrate similar fluctuations in larval viability (Wouters et al., 2001) although it has been found that by increasing the ascorbic acid (AA) content to concentrations far greater than is required for normal metabolic functioning has resulted in increased ovarian development, hatchability of eggs and larval viability (Cahu et al., 1991; Alava et al., 1993a,b; Cahu et al., 1995). The slow and selective feeding behavior of *J. edwardsii* provides challenges somewhat unique to crustaceans in providing specific concentrations of dietary components, and in particular water soluble vitamins such as AA (Conklin, 1997). AA deficiencies in crustaceans are typified by a number of gross morphological signs including retarded growth, incomplete moulting, impaired wound healing and melanised lesions beneath the exoskeleton (Hunter et al., 1979; Magarelli et al., 1979; He and Lawrence, 1993; Chen and Chang, 1994). Supplying basal AA requirements may prevent these gross morphological signs. For many crustaceans dietary amounts as little as 120 $\mu g\ g^{-1}$ are required (Conklin, 1997; He and Lawrence, 1993; D'Abramo et al., 1994; Shiau and Hsu, 1994; Giri et al., 1995) and can be supplied through a range of fresh (mussels and squid) and pellet products (Verstraete et al., 1995).

Environmental and husbandry stressors are potential causes of poor health during culture and may increase the requirement for AA (Dabrowski, 1992). Therefore the ability to maintain broodstock health is of prime importance in a captive breeding program, as is the maximization of reproductive potential. Parameters that have previously been enhanced through elevated concentrations of dietary AA have

included fecundity, egg and larval quality (Cahu et al., 1991; Alva et al., 1993a; Cahu et al., 1995).

The original form of AA used for supplementation in aquaculture feeds was L-ascorbic acid although in this form AA is easily depleted by physical factors associated with pellet production and usage, including heat, light and immersion in water (Eva et al., 1976; Grant et al., 1989). A water-stable form of AA, typically an ascorbyl polyphosphate (ApP) derivative is now widely used however, dependent upon manufacture processes, storage and the duration of water immersion even this may be subject to a degree of degradation (Giri et al., 1995; Kontara et al., 1997; Merchie et al., 1998; Moreau et al., 1998; Sangha et al., 2000). Suitability in terms of pellet stability when immersed in water is largely dependent upon the duration taken to consume the diet. The slow feeding behavior and reluctance by some *J. edwardsii* broodstock to accept formulated pellets, particularly when fresh food is also available can reduce pellet uptake. Thus the provision of a palatable AA supplement that is consumed within a relatively short period is a prerequisite for aquaculture species and crustaceans in particular.

The effect of supplementary concentrations of AA on *J. edwardsii* broodstock is unknown. The aim of this study was to examine the effect of AA supplementation via the *Artemia*-alginate pellet diet on the distribution of AA in female organs, eggs and newly hatched phyllosoma larvae, and to assess changes to indicators of broodstock and larval quality. The delivery of a stable and palatable AA supplement was achieved by using *Artemia* biomass enriched with ascorbyl 2-phosphate (A2P) (Smith et al. in press) encapsulated within an alginate pellet. Using this method AA is incorporated into *Artemia* tissue at high doses, affording it a degree of protection from degradation while providing a palatable supplement to the broodstock.

7.3. Materials and Methods

7.3.1. Broodstock collection and holding

Experiments were carried out at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories (TAFI, MRL), Hobart, Australia. Broodstock were captured from Tasmania's west coast (43° 19' S, 145° 52' E) and held at the facility for 7 months prior to the commencement of the AA dietary study in January 2002. A total of 87 females were weighed (mean ± sem; 561.7 ± 6.3 g), measured (carapace length) (104.1 ± 0.4) and stratified on body weight and carapace length. Thirty-six males were also randomly distributed among the treatments (710.6 ± 10.8 g; 112.1 ± 0.6 mm). The study consisted of 4 treatments in triplicate, with each replicate containing 10 animals (7 females, 3 males). Three additional females were utilized as an initial (Time 0) sample for biochemical analysis.

Animals were held in 12 x 600 L square fiberglass tanks under ambient conditions of light and temperature. Light intensity at the tank floor was < 4 μmol s^{-1} m^{-2} during light exposure, while temperatures ranged from a low of 9.5°C in winter to 18.0°C in summer. Water supply was flow-through seawater at 300 L h^{-1}, providing 100% water exchange every 2 h. Shelter was provided in each tank by concrete blocks arranged to provide a series of shelters. All tanks were visually isolated from the rest of the facility behind a framework covered with black plastic sheeting.

7.3.2. Broodstock dietary regime

Prior to the commencement of the broodstock trial all animals were fed a basal diet consisting of fresh mussels (*Mytilus edulis*), squid (*Nototodarus sloanii*) and penaeid compound diet (Vital prawn pellet, Higashimaru, Japan) 2:2:1 dry weight (dw). The broodstock feed regime provided a range of AA concentrations primarily altered by the addition of AA-enriched and non-enriched *Artemia* biomass to the feed regime. The study commenced on 6 March 2002 and continued for 10 weeks

during the maturation period and immediately prior to the pre-ovigerous moult. Broodstock were fed one of four dietary regimes consisting of:

- B – Basal diet; fresh mussels, squid and penaeid compound diet (2:2:1dw) – containing ≈ 150 μg AA g^{-1} dw (unpublished data), no *Artemia*
- BA_{low} – Basal diet + unenriched *Artemia*-alginate pellets (A_{low}) ≈ 150 μg AA g^{-1} dw
- BA_{high} - Basal diet + *Artemia*-alginate pellets enriched with AA (A_{high}), total diet ≈ 1350 μg AA g^{-1} dw
- BA_{med} – Basal diet + mix of A_{low} and A_{high} *Artemia*-alginate pellets, total diet ≈ 450 μg AA g^{-1} dw.

All diet/supplement combinations were fed at a weekly rate of 1.35% dw of broodstock wet weight (ww), the concentration of AA utilized in this study was within the range of other successful supplementation studies with crustaceans (Cahu et al., 1991; Sangha et al., 2000). To counter preferential consumption by spiny lobsters different dietary components were fed on separate days. Squid was fed on Monday, penaeid compound diet on Wednesday and mussels on Friday. The B dietary regime had the penaeid compound diet ration also divided between Tuesday and Thursday, as was the appropriate mix of enriched *Artemia*-alginate pellets to $BA_{low, med}$ and $_{high}$. Feed was placed on trays located on the tank bottom in the evening with any uneaten food removed within 24 h. Broodstock dietary components (10g ww) were sampled for AA analysis at time 0, 1, 6 and 24 h after immersion in seawater (35‰, 18ºC), both at the commencement and completion of study. Pellets were rinsed 3 times in 100 ml of freshly prepared 0.5 M ammonium formate, wrapped in aluminum foil, before storage in liquid nitrogen until sample analysis.

7.3.3. Preparation of AA enriched Artemia pellets

The graduated supply of AA was facilitated by the use of AA-enriched and non-enriched *Artemia* biomass incorporated into alginate pellets at various rates and used in combination with other basal dietary components (squid, mussels and prawn pellet). *Artemia* biomass was prepared twice weekly using 1.5 - 2.5 mm juvenile

Artemia that were enriched for a 24 h period with ascorbyl-2-monophosphate (A2P), a particulate form of AA (Stay C, Roche Vitamins Australia Ltd., Sydney) (Smith et al. in press). Briefly, *Artemia* culture and enrichment was conducted in 250 L cones containing aerated 1.0 µm filtered seawater maintained at 34 ± 1‰ and 28 ± 1°C. The A2P ration (final concentration 0.6 g L^{-1}) was blended (household blender) in seawater for 1 min and added to the enrichment vessel at 0 and 12 h providing an *Artemia* AA enrichment content of approximately 10 000 µg g^{-1} dw after 24 h (Smith et al., in press). A second group of *Artemia* was maintained under similar conditions but without enrichment. At the completion of 24 h, *Artemia* were siphoned onto 250 µm screens, rinsed with fresh water and held at 4°C. For *Artemia*-alginate pellet manufacture, combinations of enriched and unenriched *Artemia* were used to provide different AA concentrations; they were mixed (1:1 ww) with 12% alginate (Merck Pty Ltd, Australia) and a small amount of coarse sand to ensure negative buoyancy. The mixture was extruded through a 7 mm diameter tube into a 4% calcium chloride bath left to set for 1 min before cutting into 15 mm long pellets, draining and holding at 4° C. Care was taken to ensure air was not trapped in the mixture as this caused the pellets to float.

7.3.4. Broodstock sampling and monitoring

Female broodstock were randomly selected from each treatment in triplicate at the commencement and the completion of the 10-week feeding trial. From these animals samples of the digestive gland, ovary and tail muscle were taken for analyses. Prior to dissection broodstock were placed in ice slurry for 1 h to obtain a chill coma, samples collected, rinsed and stored for AA analysis as previously described.

Broodstock were checked daily for mortalities, moults and ovigerous females. No mortalities were recorded during feeding or the 5-month duration of egg incubation. A 2 g sample of eggs was removed from ovigerous females within 1 week of egg extrusion, rinsed and stored as previously described.

7.3.5. Phyllosoma collection and treatments

Prior to hatching females were removed from the dietary treatment tank and placed into individual 20 l hatching containers suspended in the broodstock-hatching tank and supplied with isothermal water (Smith et al., 2003a). At hatch, phyllosoma larvae were skimmed daily from the surface of the hatching container and counted. Cumulative totals were tallied over the typical 4-5 day hatch period for each female and a calculation of relative viable fecundity made. Relative viable fecundity is a measure of the total number of phyllosoma hatching from each female divided by female carapace length; to standardize the phyllosoma number/female size relationship. It does not include non-viable eggs or larvae that do not progress beyond the naupliosoma stage, which is the brief 0.5 – 1 h post-hatch stage (Tong et al., 2000).

Phyllosoma from the 2nd day of hatch were randomly allocated to the following treatments:

- Sample for analysis of AA. A 2 g sample of Stage I phyllosoma was taken for AA analysis, rinsed and stored as previously described.
- Measurements taken of newly hatched Stage I larvae. The body length (anterior tip of the cephalothorax to the posterior point of the abdomen) of 20 newly-hatched Stage I phyllosoma from each female were measured at hatch and at Stage II (day 14), as described by Lesser (1978). Measurements were obtained using an overhead projection microscope (Nikon profile projector, model 6C, 20 times magnification).
- Survival of larvae in short-term temperature and salinity challenges (activity test). This test assesses the viability of phyllosoma according to the modified method of Smith et al. (2003b) (21°C used instead of 23°C). A lower stress index is indicative of higher survival in culture.
- Culture of larvae for 14 d (Stage II) with measures of total length and survival taken. Phyllosoma larvae were cultured to Stage II (14 d) in 1 L glass beakers (in triplicate) containing gently aerated seawater (1. 0 µm filtered, 34 ± 1‰, 18 ± 0.5°C). Growth, survival and stress indices were monitored during this period. Phyllosoma larvae in all treatments were fed 1.5mm unenriched juvenile

Artemia containing approximately 150 µg AA g^{-1} dw at a rate of 3 ml^{-1} day^{-1}. This feed coincided with total water exchange in the beakers, flushing away uneaten *Artemia* and addition of antibiotics (Oxytetracycline hydrochloride 25ppm, Engemycin 100, Intervet, Australia) to the culture water. During Stage I, larval densities in all treatments were 100 larvae L^{-1} $replicate^{-1}$. Treatments were terminated at Day 14 with larval size and survival recorded.

7.3.6. Ascorbic acid analysis

Artemia and phyllosoma samples were freeze-dried for 24 h (Dynavac freeze-drier F.D.3, -80°C, 33x 10^{-3} Mbar), dw taken and 50 - 100 mg subsamples set aside for analysis. Ascorbic acid was extracted from subsamples using metaphosphoric acid (3%) + acetic acid (8%) (MPA) by the method of Brown and Miller (1992), with minor modifications to the sonication step (sonicated once only for 30 sec). Total AA (sum of ascorbic acid plus dehydroascorbic acid) was detected using this assay but not AA in the form of A2P. However, a preliminary trial demonstrated that there was minimal additional AA present in *Artemia* post enrichment when using a phosphatase incubation step to convert A2P to AA (Wang et al., 1988). HPLC analysis was conducted on 50 µl samples using a Waters Model 600E liquid chromatograph system. The derivatized product was detected using a Waters Model 475 scanning fluorescence detector; the excitation maximum was set at 355 nm and emission maximum at 425 nm. The peak area was quantified using Waters Millennium software. The column used was a C18 Novapak, Waters; 3.9 x 150 mm, which was eluted isocratically with 80:20 (v/v) 0.08 M potassium dihydrogen phosphate (pH 7.8) and methanol, respectively, at a flow rate of 0.8 ml min^{-1}. The results were expressed in µg AA g^{-1} dw.

7.4. Statistical analyses

Statistical analyses were conducted using regression and correlation analysis, one-way analysis of variance with Tukey-Kramer HSD tests used for post-hoc comparison. Analysis of covariance was used to discern differences between slopes and regression intercepts. Arcsin√ transforms were performed on percentage data.

Unless stated otherwise, $P < 0.05$ was considered significantly different (Sokal and Rohlf, 1995). Data are presented as mean ± sem. Statistics were executed using JMP version 5.1 (SAS Institute Inc.).

7.5. Results

7.5.1. Broodstock diet leaching

At Time 0, AA enriched *Artemia*-alginate pellets (A_{high}) contained 9 153 μg AA g^{-1} dw, the ration was adjusted for the AA inclusion of diet BA_{high} (1350 μg g^{-1}). After 1 h immersion in water the AA content of the pellets was reduced by 34% to 6039 μg g^{-1}, with concomitant decreases to the AA dietary concentration in BA_{high} (900 μg g^{-1}). Immersion of A_{high} *Artemia*-alginate pellets in seawater for 6 and 24 h resulted in 81 and 97% reductions to their AA content (Table 7.1). At Time 0, A_{med} enriched *Artemia*-alginate pellets contained 2 299 μg g^{-1} dw, the ration was adjusted to provide the required inclusion for diet BA_{med} (450 μg g^{-1}). After 1 h immersion in water the AA content of the pellets was reduced to 1555 μg g^{-1}, with concomitant reductions to the AA dietary concentration in BA_{med} (300 μg g^{-1}). Immersion of A_{med} *Artemia*-alginate pellets in seawater for 6 and 24 h resulted in 70 and 91% reductions to their AA content. At Time 0, A_{low} enriched *Artemia*-alginate pellets contained 150 μg g^{-1} dw. After 1 h immersion in water the AA content of the pellets was reduced to 142 μg g^{-1}, with concomitant reductions to the AA dietary concentration in BA_{low} (136 μg g^{-1}). Immersion of A_{low} *Artemia*-alginate pellets in seawater for 6 and 24 h resulted in 48 and 79% reductions to their AA content. On the majority of days, >80% of AA enriched pellets appeared to be consumed within the first hour with10% of the remainder consumed over the next 6 h (all dietary components were fed at least 10% in excess of consumption).

Table 7.1 Ascorbic acid (AA) content in *Artemia* enriched alginate pellets immersed in seawater, AA is presented both quantitatively (± sem) and as a % loss, n = 3. B – Basal diet; fresh mussels,

squid and penaeid compound diet (2:2:1dw), BA_{low} – Basal diet + unenriched *Artemia*-alginate pellets (A_{low}), BA_{high} - Basal diet + *Artemia*-alginate pellets enriched with AA (A_{high}), BA_{med} – Basal diet + mix of A_{low} and A_{high} *Artemia*-alginate pellets.

Exposure period	Ascorbic acid retention and % loss after exposure to water					
	BA_{low}		BA_{med}		BA_{high}	
	AA μg g^{-1}	% loss	AA μg g^{-1}	% loss	AA μg g^{-1}	% loss
Basal content	156 ± 12	0	2299 ± 110	0	9150 ± 261	0
After 1h	142 ± 6	9	1555 ± 3	32	6050 ± 130	34
After 6h	81 ± 5	48	692 ± 174	70	1708 ± 665	81
After 24h	32 ± 5	79	200 ± 130	91	276 ± 165	97

7.5.2. Broodstock organ concentrations

The digestive gland in Time 0 samples weighed ≈ 25 g ww and was unchanged by feed duration or treatment. Ovarian ww was initially 24.5 ± 0.7 g, increasing to a pooled mean of 35.5 ± 3.2 g at the completion of the study (not significantly different between treatments). Total tail muscle ww was unchanged, 241.7 ± 4.5 and constituted 43% of total ww of 562 g animals (sample mean). On this basis the AA contribution of the digestive gland, ovary and tail muscle a total organ-tail muscle AA pool was calculated (Table 7.2). The digestive gland contained 20% of the AA pool prior to enrichment, remained relatively stable in all treatments except for BA_{high} where significant increases were evident. The ovary contained 36% of the AA pool prior to enrichment increasing to become the dominant AA store during the period of the feed study, particularly in the B and B_{low} treatments with 54 and 61% of all AA located in the ovary. Before enrichment the tail muscle made the greatest contribution to the organ-tail muscle AA pool of 44%, after enrichment this was significantly less, between 21-28% across all treatments.

The concentration of AA in the digestive gland was unchanged with feeding B or BA_{med}. Animals fed BA_{low} experienced a significant reduction in AA while those that received BA_{high} experienced > 3 fold increases (Fig. 7.1a). The constant size of the digestive gland meant that the total content of AA in the digestive gland displayed the same trends as seen in the concentration data. The AA concentration in the ovary altered in a treatment specific manner; animals underwent minor decreases when fed B, were unchanged in BA_{low} and increased significantly when fed BA_{med} or BA_{high} (Fig 7.1b). In general the size of the ovary increased during the study however the inconsistent nature of the increases resulted in no significant

difference in total AA across all treatments even though AA content increased from 3657 μg at the commencement of the study to 4804, 5759, 6110 and 8731μg in animals fed diets B, $_{low}$, $_{med}$ and $_{high}$, respectively.

The tail muscle underwent significant alterations to AA incorporation with feeding and maturation (Fig 7.1c). The AA tail muscle concentration was unchanged in animals fed $BA_{high,}$ reduced by 25% in BA_{med} fed animals and by ≈ 45% in animals subject to treatments B and $BA_{low.}$ The contribution of tail muscle AA to the organ/tail muscle pool decreased from 44 – <29% with feeding.

Table 7. 2 Distribution of ascorbic acid (AA) in the organ (digestive gland, ovary) /tail muscle pool of *J. edwardsii* females before and after feeding an ascorbic acid supplemented diet for 10 weeks (See Table 1. for details) Data was analysed using ANOVA, $P<0.05$ was considered significant. B – Basal diet; fresh mussels, squid and penaeid compound diet (2:2:1dw), BA_{low} – Basal diet + unenriched *Artemia*-alginate pellets (A_{low}), BA_{high} - Basal diet + *Artemia*-alginate pellets enriched with AA (A_{high}), BA_{med} – Basal diet + mix of A_{low} and A_{high} *Artemia*-alginate pellets.

	Relative contribution (%) to organ/tail muscle AA pool				
		After feeding for 10 weeks			
Organs	Time 0	B	BA_{low}	BA_{med}	BA_{high}
Digestive gland	20 [b]	18 [b]	13 [b]	20 [b]	37 [a]
Ovary	36 [b]	54 [a]	61 [a]	52 [a]	42 [b]
Tail muscle	44 [a]	28 [b]	26 [b]	28 [b]	21 [b]

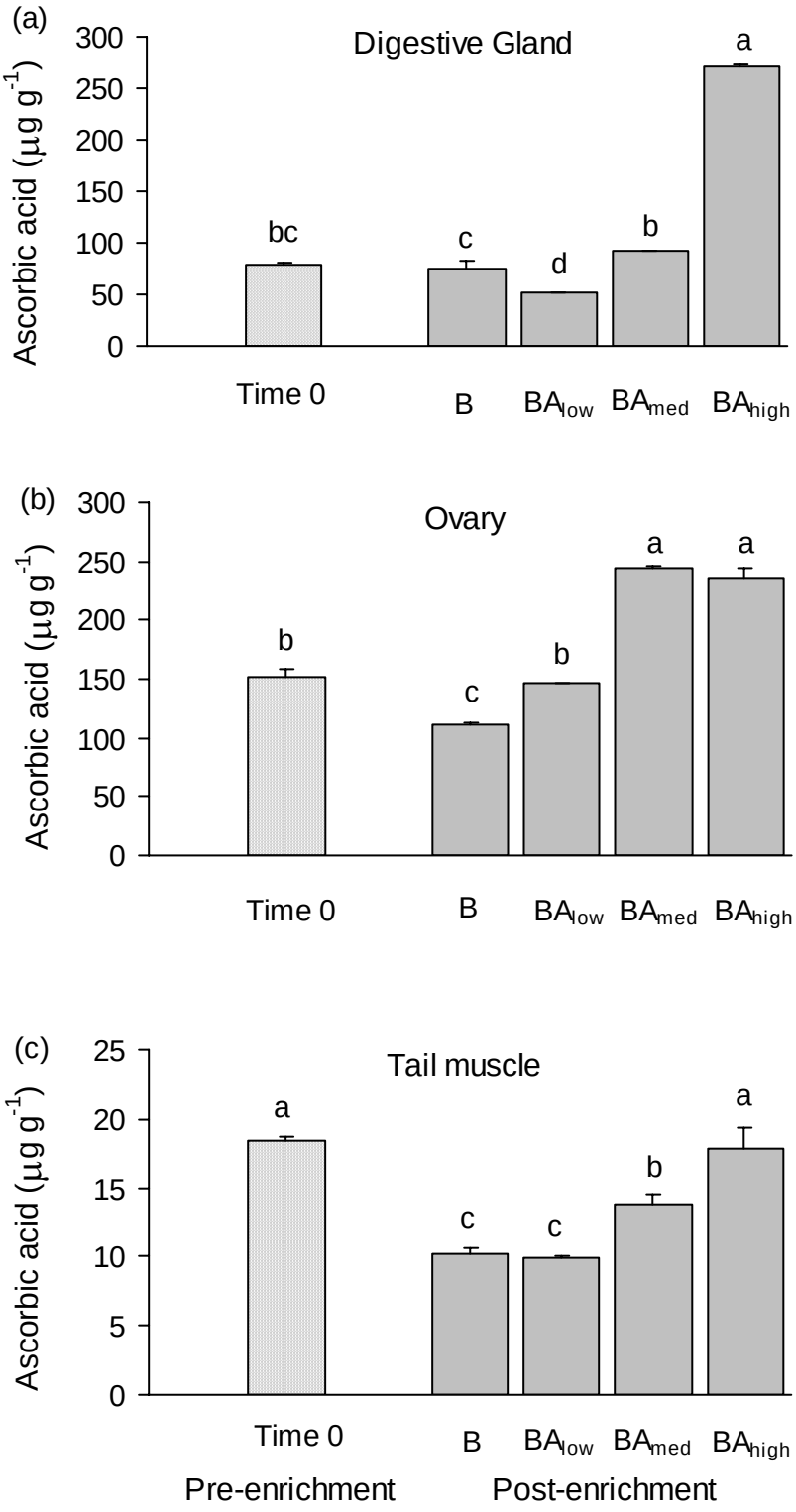

Fig. 7.1 Tissue concentrations of ascorbic acid (AA µg g^{-1} dw) prior to and after feeding supplementary AA for 10 weeks. All treatments included basal (B) fresh and compound prawn diet and were supplemented with *Artemia* pellets (comprising unenriched and/or AA-enriched *Artemia*) to provide dietary AA concentrations of 150 (BA_{low}), 450 (BA_{med}) or 1350 ($BA_{hig\ h}$) µg g^{-1}. Different letters denote a significant difference between tissue concentrations of AA (ANOVA, P<0.05). Each treatment was examined in triplicate.

During this study there were numerous changes to the distribution of AA within the digestive gland, ovary and tail muscle compared to Time 0. In animals receiving ≤ 450 μg AA g^{-1} (i.e., B, $BA_{low, med}$), increases to the ovarian AA content were accompanied by concomitant reductions to the AA content in tail muscle, and to a small degree the digestive gland (BA_{low}) (Fig. 7.2). In animals receiving BA_{high} both the digestive gland and ovary experienced large increases to total AA while tail muscle maintained Time 0 concentrations.

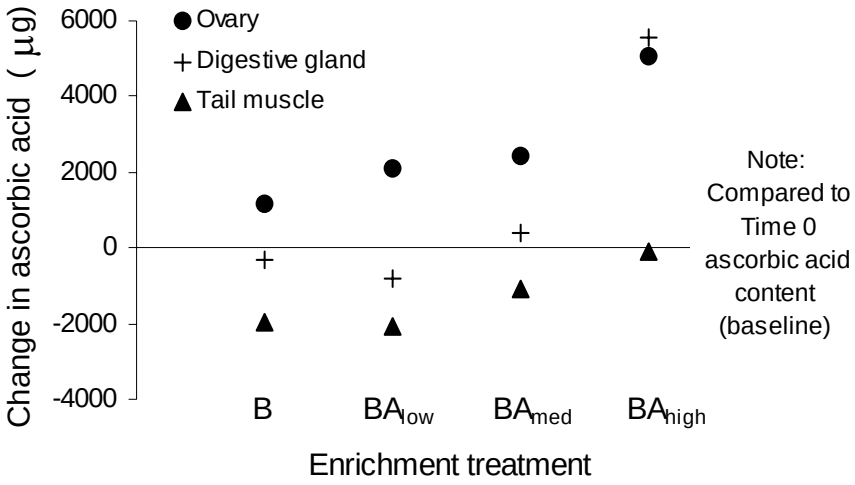

Fig. 7.2 Change in total ascorbic acid (μg) in the digestive gland, ovary and tail muscle of *Jasus edwardsii* relative to the content at Time 0 (baseline of plot). All treatments included basal (B) fresh and compound prawn diet and were supplemented with *Artemia* pellets (comprising unenriched and/or AA-enriched *Artemia*) to provide dietary AA concentrations of 150 (BA_{low}), 450 (BA_{med}) or 1350 ($BA_{hig\ h}$) μg g^{-1}. Each treatment was examined in triplicate.

7.5.3. Egg and phyllosoma concentrations

Eggs derived from broodstock fed diet B contained the lowest AA concentration of 164 μg g^{-1} and this concentration increased to a maximum of 216 μg g^{-1} for the highest AA supplementation, BA_{high} (Fig 7.3). The pattern of endogenous AA in newly-hatched Stage I phyllosoma was similar to that of the eggs, with BA_{high} phyllosoma containing higher concentrations of AA (207 μg g^{-1}) than those of phyllosoma from other treatments (< 164 μg g^{-1}). There was a significant

relationship between initial dietary AA concentration and the concentration of AA in that of eggs or phyllosoma (Fig 7.4a) and a similar relationship after 1 h immersion of the feed in seawater, (i.e., when ≈ 80% of pellets were consumed) (Fig 7.4b). The regression slopes between initial AA dietary concentrations and the concentration of AA in egg and phyllosoma did not differ ($F_{2,13}$ = 0.9130; *P* = 0.4323) nor did the intercepts ($F_{1,14}$ = 1.0047; *P* = 0.4003). Similarly, the AA content of diets post 1 h immersion in water did not alter the slope ($F_{2,13}$ = 0.9130; *P* = 0.4323) or intercept ($F_{1,14}$ = 0.3332; *P* = 0.5771). A significant relationship existed between the concentration of AA in eggs and phyllosoma (R^2 = 0.4341; Prob>F 0.0055; phyllosoma = 38.7048 + 0.6940*egg).

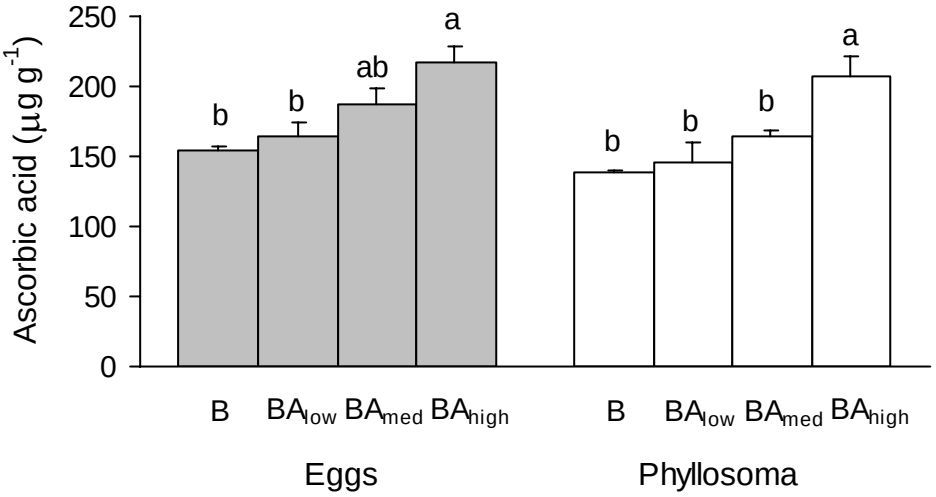

Fig. 7.3 Egg and phyllosoma ascorbic acid (AA) concentrations resulting from different amounts of broodstock AA supplementation. All treatments included basal (B) fresh and compound prawn diet and were supplemented with *Artemia* pellets (comprising unenriched and/or AA-enriched *Artemia*) to provide dietary AA concentrations of 150 (BA_{low}), 450 (BA_{med}) or 1350 ($BA_{hig\ h}$) µg g^{-1}. Different letters denote a significant difference between treatments in eggs or phyllosoma (ANOVA, *P*<0.05). Each treatment was examined in triplicate.

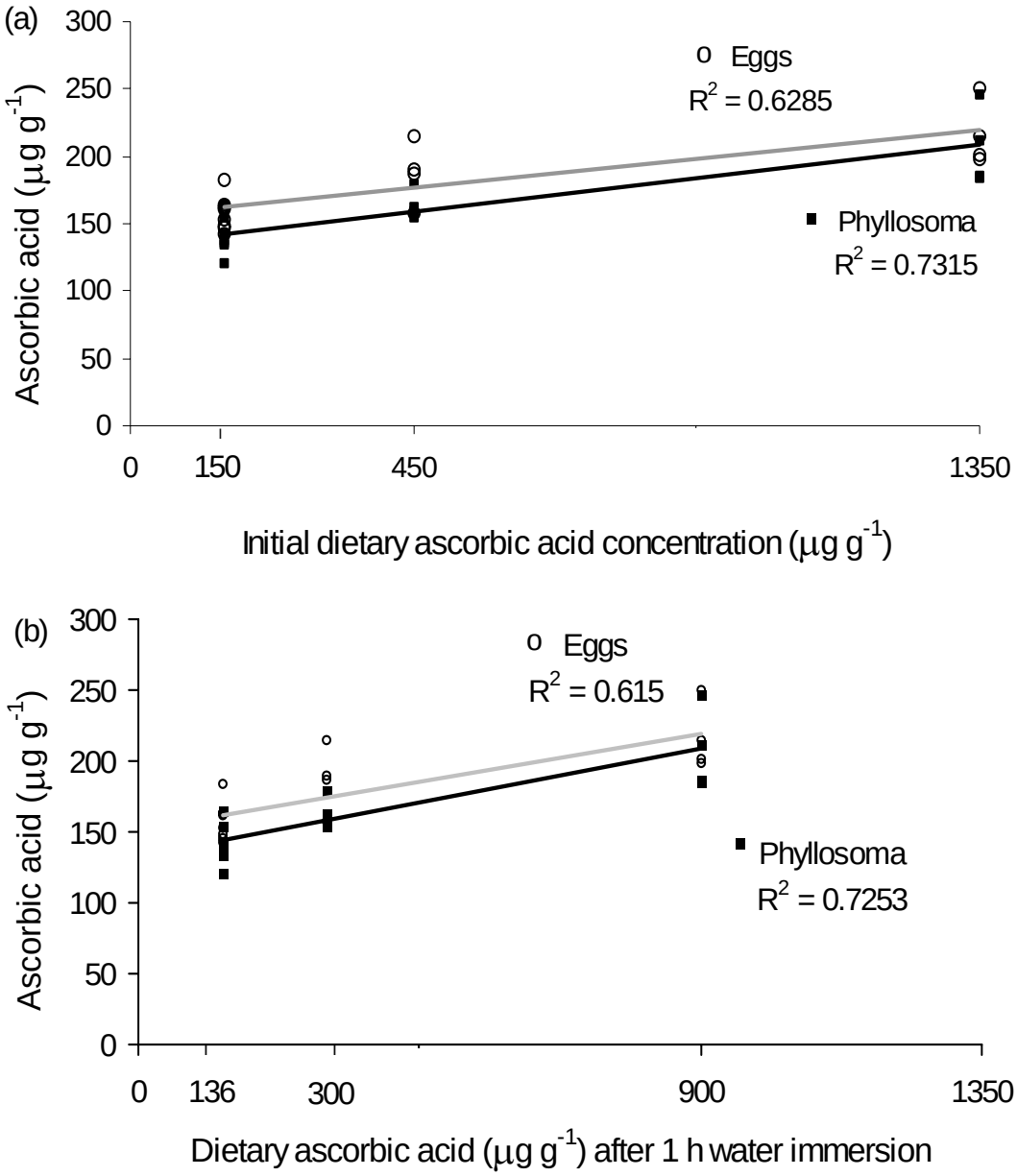

Fig. 7.4 The relationship between (a) dietary ascorbic acid (AA) and egg or phyllosoma AA, and (b) dietary AA after 1 h immersion in seawater and egg or phyllosoma AA concentrations. All treatments included basal (B) fresh and compound prawn diet and were supplemented with *Artemia* pellets (comprising unenriched and/or AA-enriched *Artemia*) to provide dietary AA concentrations of 150 (BA_{low}), 450 (BA_{med}) or 1350 ($BA_{hig\ h}$) µg g^{-1}. Regression analysis determined the significance of relationships with analysis of covariance used to determine differences between the slopes and intercepts of the lines. Four replicates were examined for egg or phyllosoma dietary concentration.

7.5.4. Fecundity, egg and phyllosoma size/survival

There was no difference in the relative viable fecundity of animals from the different dietary treatments, although AA supplementation reduced the variability of the data as dietary AA increased (Fig. 7.5). Supplementation of the broodstock diet with AA did not significantly reduce the stress index, even at the highest concentration, and had no effect on phyllosoma size or that of phyllosoma survival (Table 7.3). There was a significant negative correlation between phyllosoma stress indices and survival in culture (R = -0.6463; Prob>F 0.0068).

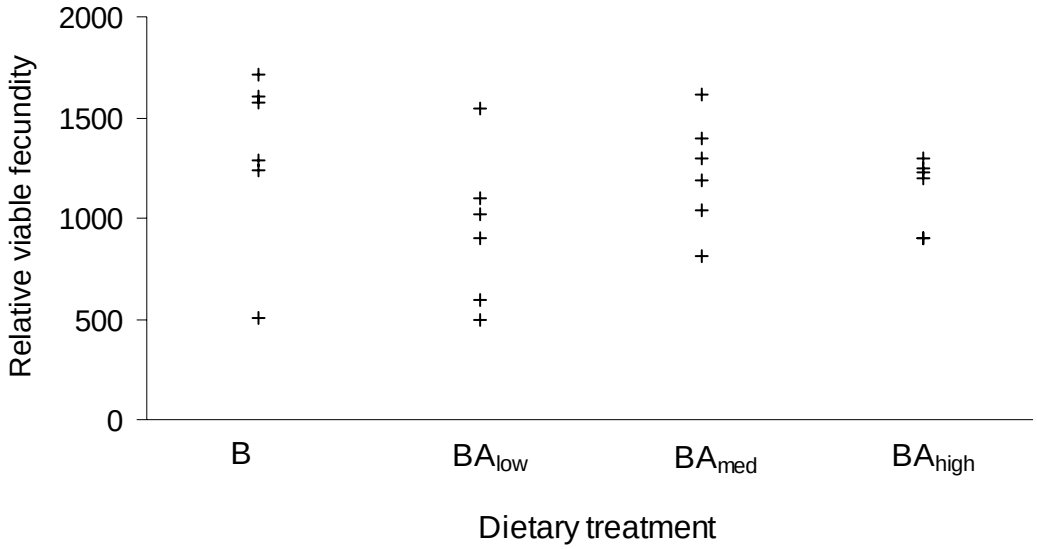

Fig. 7.5 The relationship between relative viable fecundity (total number of phyllosoma produced adjusted for female size) and broodstock dietary treatment. All treatments included basal (B) fresh and compound prawn diet and were supplemented with *Artemia* pellets (comprising unenriched and/or AA-enriched *Artemia*) to provide dietary AA concentrations of 150 (BA_{low}), 450 (BA_{med}) or 1350 ($BA_{hig\ h}$) μg g^{-1}, n = 6 per treatment.

Table 7.3 *Jasus edwardsii* phyllosoma quality measures of stress indices, size (total length, mm) and survival (%) after supplementation of broodstock diets with AA. No significant difference was noted across any quality parameter. B – Basal diet; fresh mussels, squid and penaeid compound diet (2:2:1dw), BA_{low} – Basal diet + unenriched *Artemia*-alginate pellets (A_{low}), BA_{high} - Basal diet + *Artemia*-alginate pellets enriched with AA (A_{high}), BA_{med} – Basal diet + mix of A_{low} and A_{high} *Artemia*-alginate pellets.

Quality measure	Broodstock ascorbic acid treatments			
	B	BA_{low}	BA_{med}	BA_{high}
Stress indices	36.4 ± 18.3	38.1 ± 16.2	35.5 ± 12.7	20.9 ± 8.0
Size Stage I	2.15 ± 0.02	2.10 ± 0.02	2.15 ± 0.01	2.15 ± 0.03
Size Stage II	3.00 ± 0.01	3.04 ± 0.03	3.02 ± 0.03	3.01 ± 0.04
Survival Stage II	80.9 ± 5.4	83.8 ± 6.4	66.4 ± 18.7	79.5 ± 2.0

7.6. Discussion

7.6.1. Broodstock diet leaching

Enrichment of *Artemia* with A2P and the subsequent conversion and maintenance as L-ascorbic acid (Smith et al., in press) allows high concentrations of AA to be delivered in the diet via an alginate package. While L-ascorbic acid is easily assimilated by crustaceans it is not generally the dietary AA source of choice due to rapid leaching and oxidization associated with prolonged immersion in water before consumption (Shiau and Hsu, 1994; Kontara et al., 1997). During this study, L-ascorbic acid (fed to *Artemia* as A2P but incorporated as L-ascorbic acid) was retained far in excess of previous reports in other compound diets where only 2.3% was left after 1 h (Souissi, 1991) and undetectable by 3 h (Shigueno and Itoh, 1988). *Artemia* AA alginate pellets maintained similar concentrations of AA to protected forms such as ApP (Giri et al., 1995; Kontara et al., 1997; Merchie et al., 1998; Moreau et al., 1998; Sangha et al., 2000). When supplementary AA is required and diet acceptance by animals is important we have demonstrated that *Artemia* can be enriched with high tissue concentrations of AA and that this can be maintained within that of an alginate pellet. The maintenance of AA within the pellet was of sufficient duration to allow even slow feeding species such as *J. edwardsii* to be exposed to high dietary concentrations of AA.

7.6.2. Broodstock organ concentrations

AA normally accumulates in ovarian tissue during maturation and prior to egg extrusion in crustaceans (Guary et al., 1975; Alva et al. 1993a; Sangha et al., 2000), although there are some exceptions to this pattern (Wouters et al., 2001). Animals from this study demonstrated increased ovarian AA concentrations, albeit moderate (150 – 240 µg g^{-1}), with AA supplementation (diets containing 450 and 1350 µg g^{-1}) and maintained basal ovarian concentrations sufficient to sustain viable embryonic development without supplementation (150 µg g^{-1}). Increased AA ovarian concentration was not in direct proportion to dietary input (150 – 1350 µg

g^{-1}) and total ovarian AA content did not increase by more than 160% between animals receiving low or high AA dietary supplementation while dietary concentrations increased by 900%. Increasing AA content did not result in significant improvements to larval quality, as evident in other crustaceans (Kontara et al., 1997; Moreau et al., 1998). The ovarian AA content of *J. edwardsii*, even after the highest level of supplementation was low compared to other wild caught sexually mature crustaceans, such as *Macrobrachium rosenbergii* (210 - 450 µg g^{-1}) and *Litopenaeus vannamei* (352 – 461 µg g^{-1}) (Cavalli, 2000; Wouters et al., 2001). Closer examination of the reproductive strategies of these two species illustrates that they are repeat-spawners, contrary to the annual spawning strategy of *J. edwardsii* and therefore likely to require larger AA reserves to maintain larval quality in successive spawning events (Cahu et al., 1991).

The theory that ovarian AA in crustaceans is sequestered from digestive gland stores (Guary et al., 1975; Sangha et al., 2000) is difficult to quantify from this study. There is a reduction in digestive gland stores in treatment BA_{low} and a concomitant increase in ovarian content, which supports the theory of a minor role for the mobilization of digestive gland stores to the ovary. However, in supplemented animals, AA content increases in both organs independent of each other. This suggests that while the digestive gland has a storage role it is not specific for ovarian maturation at least during the current sampling i.e., up until the pre-ovigerous moult. Following the moult approximately a 3-week period exists prior to mating and egg extrusion however, it remains unlikely that digestive gland stores would have been heavily mobilized during this period with low but graduated concentrations of AA present in eggs concomitant with graduated dietary concentrations.

The tail muscle although only containing low concentrations of AA makes a significant contribution to the combined digestive gland, ovary and tail muscle AA pool (44% before enrichment) because of its large total weight. Tail muscle is generally reported as only contributing small amounts to the AA pool (Sangha et al., 2000) contrary to our experience. In animals fed the low AA diets (i.e., B and $BA_{low, med}$) there were large decreases in tail muscle AA content concomitant with increased ovarian content. This pattern of depletion and deposition suggests

sequestering of tail muscle AA stores for use in the ovary. With medium to high AA supplementation ($BA_{med, high}$) it appears the demand on tail muscle stores is reduced or negligible. It is apparent in these two treatments that, as with lipid metabolism during ovarian maturation, it may be possible to utilize dietary AA supply if present in sufficient quantities, rather than depleting specific organ stores (Clark, 1982). We speculate that the pool of AA contained in the tail muscle is readily mobilized to satisfy the requirements of maturation when dietary supply is low. A useful adaptation considering spiny lobsters are opportunistic foragers often exposed to a wide variety of food items, which may be subject to seasonal or spatial availability (Jernakoff et al., 1993; Barkai et al., 1996).

7.6.3. Egg and phyllosoma concentrations

The increasing AA content of the broodstock diet was reflected in a graduated AA content in both eggs and phyllosoma although not proportional to dietary intake. Maximum egg concentrations of 650 μg g^{-1}, equivalent to the dietary concentration, were maintained in successive spawning events in *Penaeus indicus* (Cahu et al., 1991), up to 3 times the highest AA content evident in *J. edwardsii* eggs. The content of AA in *J. edwardsii* eggs was not depleted during egg incubation to larval hatch across all treatments. This is contrary to suggestions in other crustaceans that AA content is heavily utilized during embryonic development associated with collagen synthesis (Cahu et al., 1991), specifically as a co-factor in the hydroxylation of proline to form collagen (Robertson and Schwartz, 1953; Hunter et al., 1979; Dabrowski, 1992). It is suggested that AA metabolism during embryonic development may have been restricted to some essential biological functions while more general roles such as the prevention of lipid peroxidation may have been undertaken by other nutrients such as α-tocopherol (Cahu et al. 1995).

7.6.4. Fecundity phyllosoma size and survival

AA supplementation has previously been associated with enhanced ovarian development in fish and crustaceans (Soliman et al. 1986; Alva et al. 1993b) and

improvements to egg hatchability (Cahu et al., 1991), i.e. potential determinants of relative viable fecundity. The measure of relative viable fecundity did not differ among treatments although with increasing AA supplementation, less variation was noted between replicates. The role of AA in improving consistency in relative viable fecundity may be related to its role in mediating stress response (Dabrowski, 1992). Broodstock with low concentrations of AA may demonstrate greater reproductive variability dependant upon their ability to respond to husbandry stress during maturation.

While there was a difference between AA content of phyllosoma, this did not translate into alterations in their performance in activity tests, size and survival in culture. We suggest the increase in AA content across treatments was minimal and insufficient to elicit a positive response in broodstock or phyllosoma quality. The ability to deliver high doses of AA in the diet to phyllosoma via the broodstock (and hence eggs) will be limited due to relatively small differences between the minimum and maximum ovarian AA concentrations. It was noted that AA was not significantly depleted during egg development to hatch, thus a minimum requirement is perhaps the reason for ovarian AA concentrations to be low and easily saturated.

A relationship existed between larval survival in the activity test and in culture, which was independent of phyllosoma AA concentration. If supplementary AA was likely to influence phyllosoma quality, the relatively low levels provided by ovarian saturation would not appreciably assist this process, with 10-fold increase in endogenous AA content is usually required to obtain tangible improvements (Merchie et al., 1997). Therefore, a direct route, i.e., through phyllosoma feeds, may be required. In general, younger animals (especially larvae) have higher specific metabolic rates and greater AA demands than large adults. With increased demands also comes the ability for increased uptake and storage capacity (Sandnes et al., 1992; Chen and Chang, 1994; Merchie et al., 1993; Moreau et al., 1998). Therefore, the likelihood of being able to supplement phyllosoma with higher amounts of AA and hence elicit an improvement in larval quality response is much greater than would be expected by supplementing adult broodstock.

7.7. Conclusion

This study demonstrated the ability to use an alginate-bound *Artemia* product as a means of delivering dietary supplements that may be subject to degradation providing different options to deliver a range of specific enrichments or chemotherapeutics. We demonstrated that *J. edwardsii* broodstock are able to sequester the AA required for ovarian maturation from natural dietary components and body stores, in particular from the tail muscle; previously thought to contribute little to AA stores. However, the scope for increasing embryonic content of AA to improve larval quality at hatch via broodstock diet supplementation is minimal with the requirement for AA during egg development negligible. If the potential for enhancing phyllosoma quality is to be pursued further direct AA dietary supplement to phyllosoma may be an alternative avenue of research.

7.8. Acknowledgements

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8. Chapter Eight - Phyllosoma supplement – Ascorbic acid

Submitted as: Smith, G.G., Ritar, A.J., Brown, M.R. Feeding juvenile *Artemia* enriched with ascorbic acid improves larval survival in the spiny lobster *Jasus edwardsii.* Aquaculture Nutrition, “in review”.

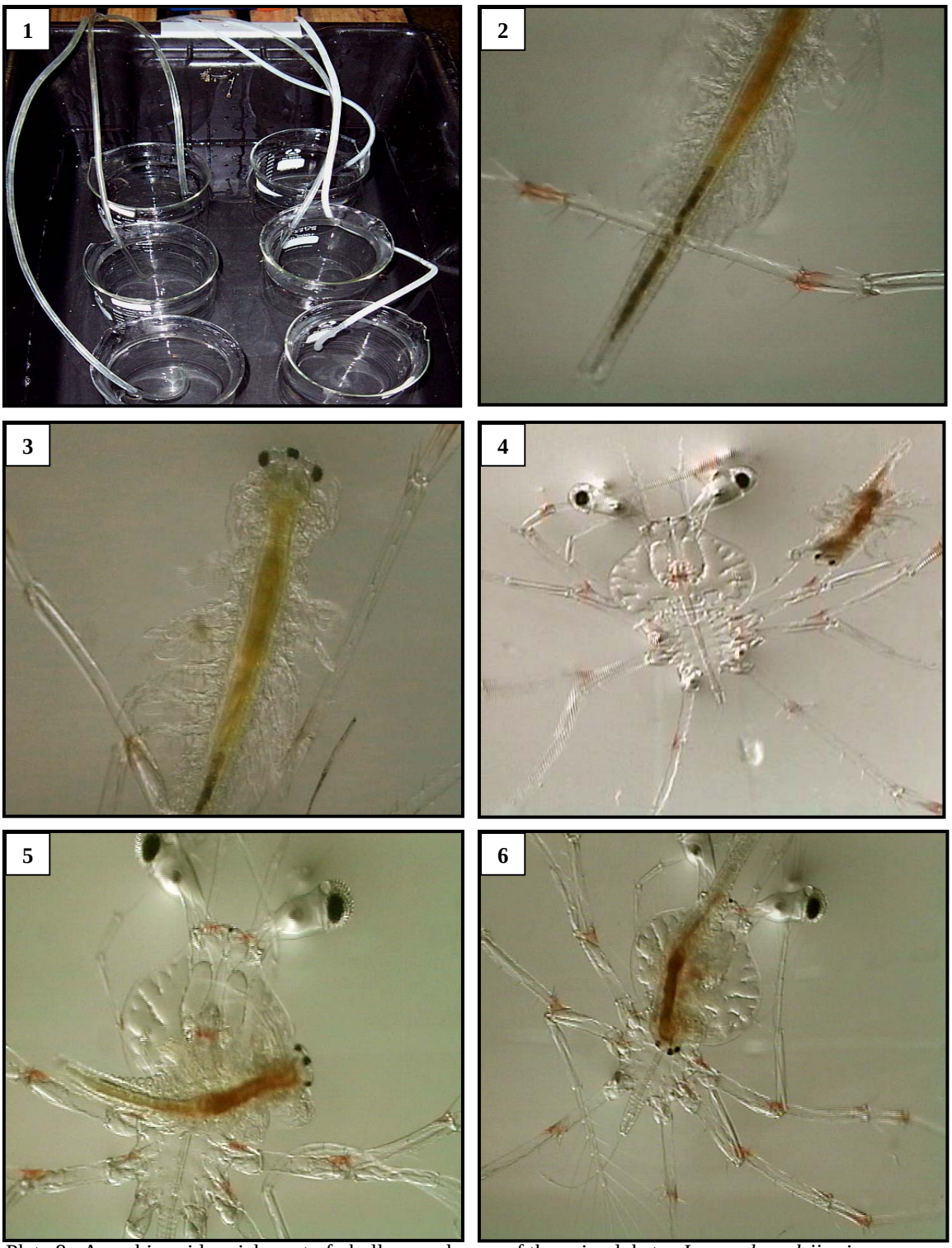

Plate 8. Ascorbic acid enrichment of phyllosoma larvae of the spiny lobster *Jasus edwardsii* using ascorbyl-2-monophosphate (A2P). (1) Culture system used to raise phyllosoma larvae; 1 L glass beakers initially stocked at 200 Stage I larvae L^{-1} fed with 3 *Artemia* ml^{-1} and treated with antibiotics (20 ppm oxytetracycline) during the daily 100% water exchange. (2, 3, 4) *Artemia* often are impaled upon spines of phyllosomas periopods before being brought towards the oral region where shredding action of the maxillipeds (5,6) allows consumption of the food particles.

8.1. Abstract

Newly-hatched *Jasus edwardsii* phyllosoma were fed unenriched *Artemia* (AA content - 166 μg g^{-1} dw), *Artemia* supplemented with algae (594 μg g^{-1} dw) or with ascorbyl-2-polyphosphate (A2P) (11 737 μg g^{-1} dw) to examine possible benefits of ascorbic acid (AA) enhancement on culture. Plain or algal-enriched *Artemia* were fed continuously for 28 days in two treatments during the study. While 4 other treatments received A2P-enriched *Artemia* on a progressive basis starting from the commencement of the trial (D-0), the 3rd (D-3), 6th (D-6) or 9th day (D-9) of Stage I (14 days) and similarly during Stage II (14 days). Prior to the commencement of A2P supplementation plain *Artemia* were supplied to these treatments. By Stage III (28 days feeding), algal, D-0 and D-3 phyllosoma had attained the largest size. The uptake and retention of AA by Stage III phyllosoma appeared to be dose-dependant with the highest level of AA incorporation evident in D-0 phyllosoma (1816 μg g^{-1} dw), while algal and plain phyllosoma contained the lowest levels (600 and 300 μg g^{-1} dw). Survival at Stage III was highest in D-0 phyllosoma (89%) and lowest in plain phyllosoma (51%). There was a positive relationship between phyllosoma AA content and larval survival (R^2 = 0.8328; P<0.0001). D-0 phyllosoma had the lowest stress index when subject to an osmotic/temperature activity test, indicative of better survival in culture compared to plain, algal and D-9 phyllosoma, which had consistently higher indices. A negative relationship existed between phyllosoma AA levels and stress indices at Stage III (R^2 = 0.9263; P<0.0001) suggesting that AA from the *Artemia* diet conferred stress resistance.

Key words: *Jasus edwardsii*, *Artemia*, ascorbic acid, ecdysis, activity tests, survival.

8.2. Introduction

Interest in the spiny lobster *Jasus edwardsii* as a candidate for aquaculture has developed as the result of fully exploited fisheries, increased consumer demand, and high product value (Schaap, 1997). To enable the aquaculture potential of this species to be fully realized the reliable closure of the life-cycle must be attained. Closure of the life-cycle of *Jasus edwardsii* is hindered by poor survival during larval culture, a prolonged phase encompassing 11 stages and numerous instars taking up to 300 days to complete (Kittaka, 1994).

An important aspect of larval culture is the ability to provide feeds that are able to sustain growth and development. Hence, the formulation of specific dietary supplements designed to overcome nutritional or physiological deficiencies that may prevent or seriously stifle the successful culture of the target species (Magarelli et al. 1979; Sandnes 1984; Léger et al. 1987). Since Léger et al. (1987) described a protocol for enrichment of *Artemia*, both nauplii and juveniles have been used as vehicles for the delivery of a wide range of products to freshwater and marine larvae. The range of enrichment products includes lipid emulsions (Léger et al. 1987; McEvoy et al. 1996; Estévez et al. 1998; Narciso et al. 1999), vitamins (Merchie et al. 1995a; Kolkovski et al. 2000) and chemotherapeutics (Skjermo et al. 1995; Touraki et al. 1996; Verlhac et al. 1996). One essential vitamin, ascorbic acid (AA) transcends these latter two classes.

Ascorbic acid is a co-factor in the hydroxylation of proline to form collagen (Robertson & Schwartz 1953; Hunter et al. 1979; Dabrowski 1992a), which is an essential component in the bones and cartilage of fish and connective tissue of crustaceans (Magarelli et al. 1979; Sato et al. 1983; Dabrowski 1990,1992b). Accordingly, the requirement for AA appears to be greatest during early life stages where specific growth rate is high (Sandnes et al. 1992; Chen & Chang 1994), and in the case of crustaceans centered on ecdysis events (Merchie et al. 1993; Moreau et al. 1998). Additional benefits of AA supplementation may include improvements to larval stress and disease resistance when incorporated at dietary concentrations that saturate the tissue (Li and Lovell 1982; Liu et al. 1989; Dabrowski 1992b; Lavens et al. 1999). Boosting a live food source, such as

Artemia, with high doses of AA may allow the targeting of specific predator developmental stages including ecdysis or metamorphosis.

The traditional method of delivering AA to larval predators involves boosting the endogenous level of AA in *Artemia* with an enrichment product. Predators with a requirement for live feed in the size range of 0.8 – 1.0 mm may be fed *Artemia* nauplii enriched with an ascorbyl palmitate (AP) lipid emulsion (Merchie et al. 1995a), while larger juvenile *Artemia* (1.5 – 2.5 mm) are more effectively enriched using a particulate product (Dobbeleir et al. 1980). It is possible to boost the AA levels in juvenile *Artemia* to > 10 000 μg g^{-1} dry weight (dw) within 24 h using an ascorbyl-2-phosphate (A2P) particulate enrichment (Smith et al., in press).

This aim of this study was to examine the effect of feeding juvenile *Artemia* enriched with AA to *J. edwardsii* phyllosoma larvae either continuously or for specified periods prior to an ecdysis event. Phyllosoma were monitored for AA levels, larval growth, survival and response to activity tests (osmotic/temperature stress, Smith et al. 2003).

8.3. Materials and Methods

8.3.1. Artemia production and experimental treatments

Decapsulated *Artemia* cysts (INVE, Great Salt Lake Prime Gold) were hatched in 50 L white fiberglass cones, with all *Artemia* hatching, culture and subsequent enrichment conducted in aerated 1.0 μm filtered seawater maintained at 30 ± 1‰ and 28 ± 1°C. At 24 h, newly-hatched *Artemia* nauplii were removed from the hatching containers, rinsed in freshwater for 2 min and cultured at a density of 5 ml^{-1} in 800 L conical tanks. *Artemia* were ongrown to a length of 1.5 mm (juvenile, 5 day old *Artemia*) on a blended brine shrimp food containing primarily rice pollard, soy and wheat flour (Eyre Peninsula Aquafeeds Pty Ltd., South Australia). *Artemia* diet was added to the culture water three times daily at a rate to maintain a Secchi depth of 25–30 cm.

Juvenile *Artemia* were harvested daily for 24 h enrichment with either microalgae (*Chaetoceros muelleri*) or A2P. The phyllosoma were fed one of the following treatments:

- Unenriched *Artemia* for the duration of the study (plain).
- *Artemia* enriched with microalgae (*C. muelleri*) for the duration of the study (algal).
- *Artemia* enriched with A2P (ascorbyl-2-monophosphate, a particulate form of AA, practically insoluble in water, containing a minimum 35% AA activity, Stay C, Roche Vitamins Australia Ltd., Sydney) for the duration of the study (D-0).
- plain *Artemia* to day 3, followed by A2P enriched *Artemia* to day 14 (encompassing ecdysis to Stage II) followed by plain *Artemia* for 3 days, then A2P enriched *Artemia* to day 28 (encompassing ecdysis to Stage III) (D-3)
- plain *Artemia* to day 6 followed by A2P enriched *Artemia* to day 14 (encompassing ecdysis to Stage II), followed by plain *Artemia* for 6 days, then A2P enriched *Artemia* to day 28 (encompassing ecdysis to Stage III) (D-6)
- plain *Artemia* to day 9 followed by A2P enriched *Artemia* to day 14 (encompassing ecdysis to Stage II), followed by plain *Artemia* for 9 days, then A2P enriched *Artemia* to day 28 (encompassing ecdysis to Stage III) (D-9)

In effect, for treatments 3-6, the start of AA enrichment was staggered at 3-day intervals from the start of Stage I and similarly during Stage II. For 1-2 days prior to ecdysis in all treatments, both Stage I and II phyllosoma exhibit non-feeding behavior.

Artemia enrichments (45 000 x 1.5 mm *Artemia*) were conducted in 5 L plastic vessels. Algal enrichment of *Artemia* was conducted once daily for 24 h using *C. muelleri* (cell density 2.02 x 10^3 cell ml^{-1}, average of 4^{th} daily monitoring). The daily A2P enrichment ration (1.2 g L^{-1}) was divided into two equal portions, blended for 30 sec in seawater (household blender, Sunbeam, Melbourne, Australia) before passing through a 40 μm screen, and added at 8 am and 8 pm.

After 24 h, enriched and plain *Artemia* were harvested and fed to phyllosoma. The AA levels in plain, algal and A2P enriched *Artemia* were 166 ± 0.5, 594 ± 0.5 and 11 737 ± 90.0 μg AA g^{-1} dw, respectively.

8.3.2. Morphological measures

The body length (anterior tip of the cephalothorax to the posterior point of the abdomen) of 20 newly-hatched Stage I phyllosoma was measured for each sample, and again at Stages II and III (days 14 and 28) as described by Lesser (1978). Measurements were obtained using an overhead projection microscope (Nikon profile projector, model 6C, 20 times magnification).

8.3.3. Activity test

An activity test was used to assess the viability of phyllosoma according to the modified method of Smith et al. (2003) (21°C instead of 23°C). Briefly, 20 phyllosoma were placed into 200 ml sample vials containing seawater adjusted to 10‰ salinity with deionised water and maintained at 21°C, all tests were conducted in triplicate. Phyllosoma in each vial were monitored at 3 min intervals with the number of inactive animals noted. A cumulative total was obtained by adding the sub-totals recorded every 3 min for 1 h, and constituted a stress index. Thus, a large stress index indicated that animals succumbed sooner to the effects of the activity test and were likely to perform poorer in culture than animals obtaining lower stress indices.

8.3.4. Larval rearing

Phyllosoma larvae were cultured to Stage III (28 d) in 1 L glass beakers (in triplicate) containing gently aerated seawater (1. 0 μm filtered, 34 ± 1‰, 18 ± 0.5°C). Growth, survival and stress indices were monitored during this period. Phyllosoma larvae in all treatments were fed 1.5mm juvenile *Artemia* at a rate of 3 ml^{-1} day^{-1}, and this feed coincided with total water exchange in the beakers, flushing away of uneaten *Artemia* and application of antibiotics (Oxytetracycline hydrochloride 25ppm, Engemycin 100, Intervet, Australia) to the culture water.

During Stage I larval densities in all treatments were initially 200 larvae L^{-1} $replicate^{-1}$, numbers were subsequently reduced by 20 on days 3, 6 and 9 for use in activity tests. At Stage II (Day 14), larval numbers were further reduced to 70 larvae L^{-1} $replicate^{-1}$ for continuation of culture to Stage III, with excess animals utilized for Stage II AA analysis. A further 20 animals $replicate^{-1}$ were sacrificed on day 28 for use in Stage III activity tests and the remainder for AA analysis. Post sampling, all *Artemia* and phyllosoma samples were rinsed (x 3) in 0.5 M ammonium formate, wrapped in aluminum foil and stored in liquid nitrogen until analysis.

8.3.5. Ascorbic acid analysis

Artemia and phyllosoma samples were freeze-dried for 24 h (Dynavac freeze-drier F.D.3, -80°C, 33x 10^{-3} Mbar), dw taken and 50 - 100 mg subsamples set aside for analysis. Ascorbic acid was extracted from subsamples using metaphosphoric acid (3%) + acetic acid (8%) (MPA) by the method of Brown and Miller (1992). Total AA (sum of ascorbic acid plus dehydroascorbic acid) was detected using this assay, not AA in the form of A2P. However, a preliminary trial demonstrated that there was minimal additional AA present in *Artemia* post enrichment when using a phosphatase incubation step to convert A2P to AA (Wang et al. 1988). HPLC analysis was conducted on 50 µl samples using a Waters Model 600E liquid chromatograph system. The derivatized product was detected using a Waters Model 475 scanning fluorescence detector; the excitation maximum was set at 355 nm and emission maximum at 425 nm. The peak area was quantified using Waters Millennium software. The column used was a C18 Novapak, Waters; 3.9 x 150 mm, which was eluted isocratically with 80:20 (v/v) 0.08 M potassium dihydrogen phosphate (pH 7.8) and methanol, respectively, at a flow rate of 0.8 ml min^{-1}. The results were expressed in µg AA g^{-1} dw.

8.4. Statistical analyses

Statistical analyses were conducted using regression and correlation analysis, one-way analysis of variance with Tukey-Kramer HSD tests used for post-hoc comparison. Arcsin√ transforms were performed on percentage data. $P < 0.05$ were considered significantly different (Sokal & Rohlf, 1995). Data are presented as mean ± sem. Statistics were executed using JMP version 5 (SAS Institute Inc.).

8.5. Results

8.5.1. Phyllosoma growth and survival

Phyllosoma growth differed significantly between treatments (ANOVA, $P<0.05$) (Fig. 8.1). By Stage II, algal and D-0 phyllosoma were the largest while plain phyllosoma were the smallest. Phyllosoma culture to Stage III saw algal phyllosoma remaining the largest but not significantly different to D-0 or D-3 fed phyllosoma, which in turn did not differ in size from D-6, D-9 or plain phyllosoma treatments. There was no significant relationship between phyllosoma AA concentration and size during Stages II ($R^2 = 0.3338$; $P = 0.2410$) or III ($R^2 = 0.2187$; $P = 0.0788$).

Survival between treatment groups differed significantly at both Stages II and III (ANOVA, $P<0.05$) (Fig. 8.2). Survival at Stage II was highest in D-0 phyllosoma (96%) and lowest in plain (79%) phyllosoma. Continuation of phyllosoma culture to Stage III saw the highest survival in D-0 phyllosoma (89%) with sequentially lower survival in D-3 (76%), algal (68%), D-6 (64%), D-9 (58%) and plain (51%) phyllosoma treatments.

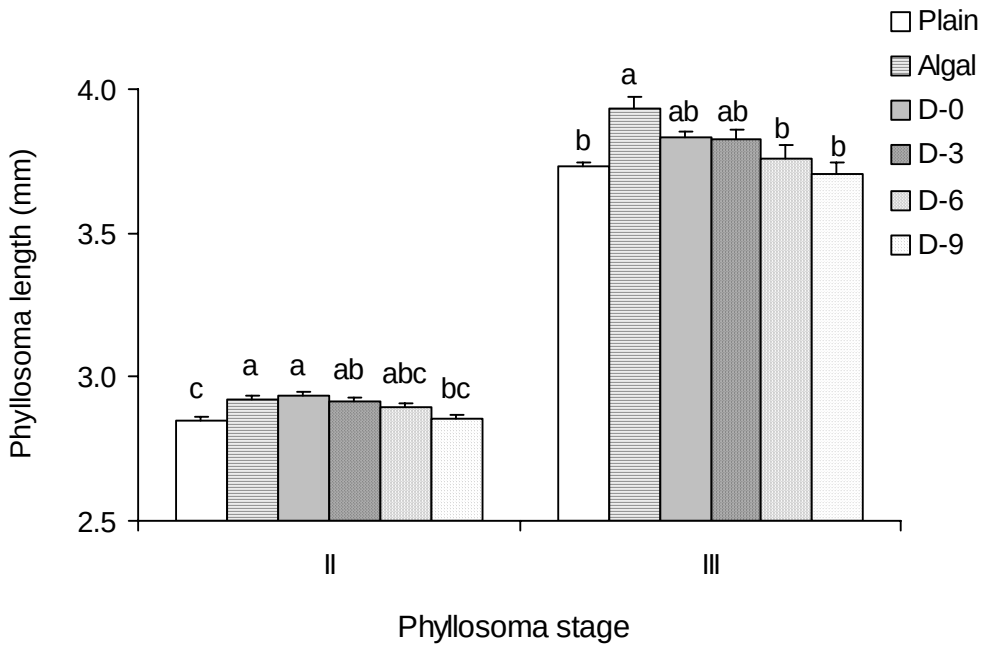

Fig. 8.1 The total length (mm) of *Jasus edwardsii* phyllosoma at Stages II and III. Phyllosoma were fed unenriched *Artemia*, *Artemia* supplemented with algae or with ascorbyl-2-polyphosphate (A2P). Plain or algal-enriched *Artemia* were fed continuously for 28 days in two treatments during the study. While 4 other treatments received A2P-enriched *Artemia* on a progressive basis starting from the commencement of the trial (D-0), the 3rd (D-3), 6th (D-6) or 9th (D-9) day of Stage I (14 days) and II (14 days). Different letters denote a significant difference between phyllosoma length (n=20 larvae) within a Stage (ANOVA, $P<0.05$).

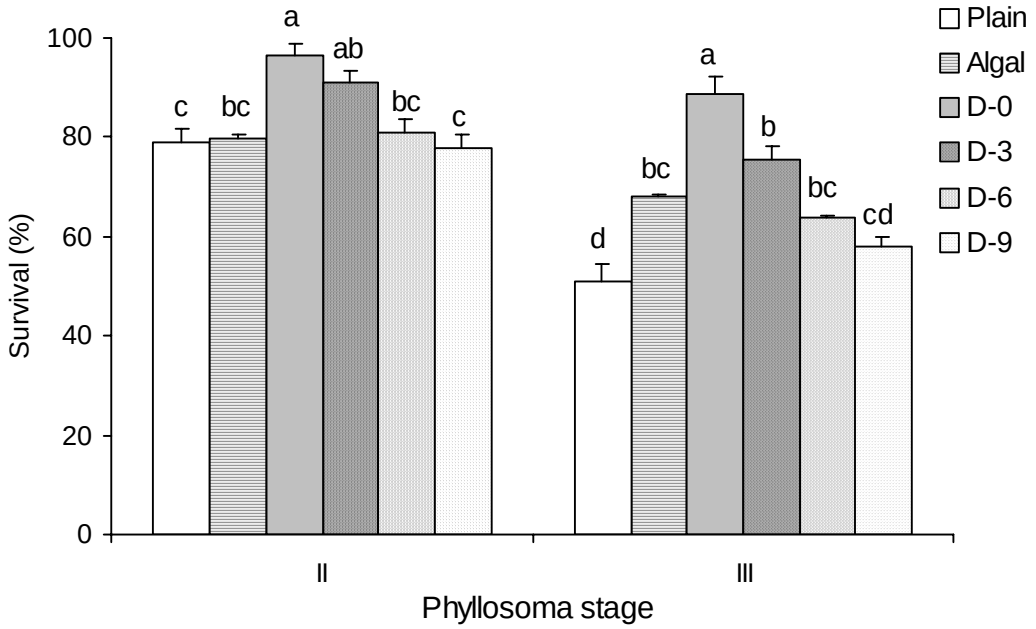

Fig. 8.2 The survival (%) of *Jasus edwardsii* phyllosoma. For feed regime, see Fig. 8.1. Different letters denote a significant difference between survival within a Stage, n = 3 culture vessels.

8.5.2. Phyllosoma AA concentration

The AA concentration in newly-hatched unenriched Stage I phyllosoma was 161 μg g^{-1}, there was a significant effect of subsequent enrichment on phyllosoma AA tissue levels (ANOVA, $P<0.05$) (Fig. 8.3). Sampling at Stage II (day 14) demonstrated an elevated concentration of AA in D-0, D-3, D-6 and D-9 phyllosoma treatments (2140 – 2250 μg g^{-1}), considerably lower levels were found in algal (510 μg g^{-1}) and plain phyllosoma treatments (380 μg g^{-1}). Culture to Stage III further altered the pattern of AA in phyllosoma treatments. The greatest level of AA was present in D-0 phyllosoma (1816 μg g^{-1}) followed by D-3 and D-6 phyllosoma (≈ 1500 μg g^{-1}) with D-9, algal and plain phyllosoma having concentrations of 1150, 600 and 300 μg g^{-1} dw, respectively.

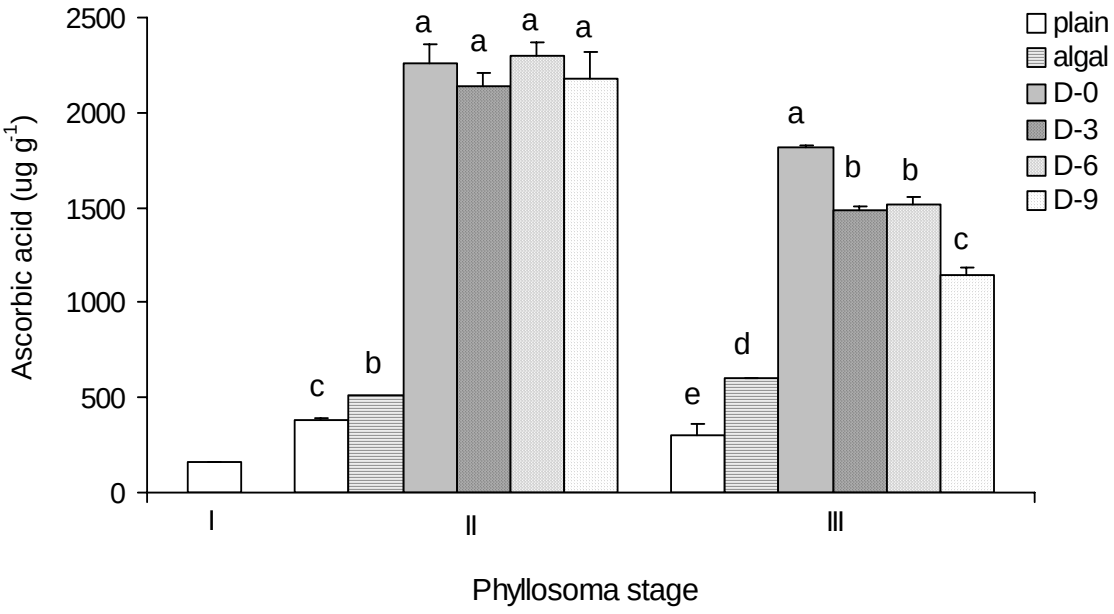

Fig. 8.3 Ascorbic acid levels (AA, μg g^{-1} dw) in phyllosoma at Stages I, II and III. For the phyllosoma feed regime, see Fig. 8.1. Sampling for phyllosoma AA levels during Stage I was conducted at hatch. Different letters denote a significant difference between phyllosoma survival (n=3 culture vessels) within a Stage (ANOVA, $P<0.05$).

There was a significant relationship between the concentration of AA in Stage III phyllosoma and their survival in culture (Fig. 8.4). Phyllosoma AA tissue concentrations of greater than 1150 μg g^{-1} resulted in increased larval survival (valid between the AA values of 300 and 1816 μg g^{-1}). Data did not include

phyllosoma fed algal-enriched *Artemia* due to additional nutritional factors supplied to this treatment group.

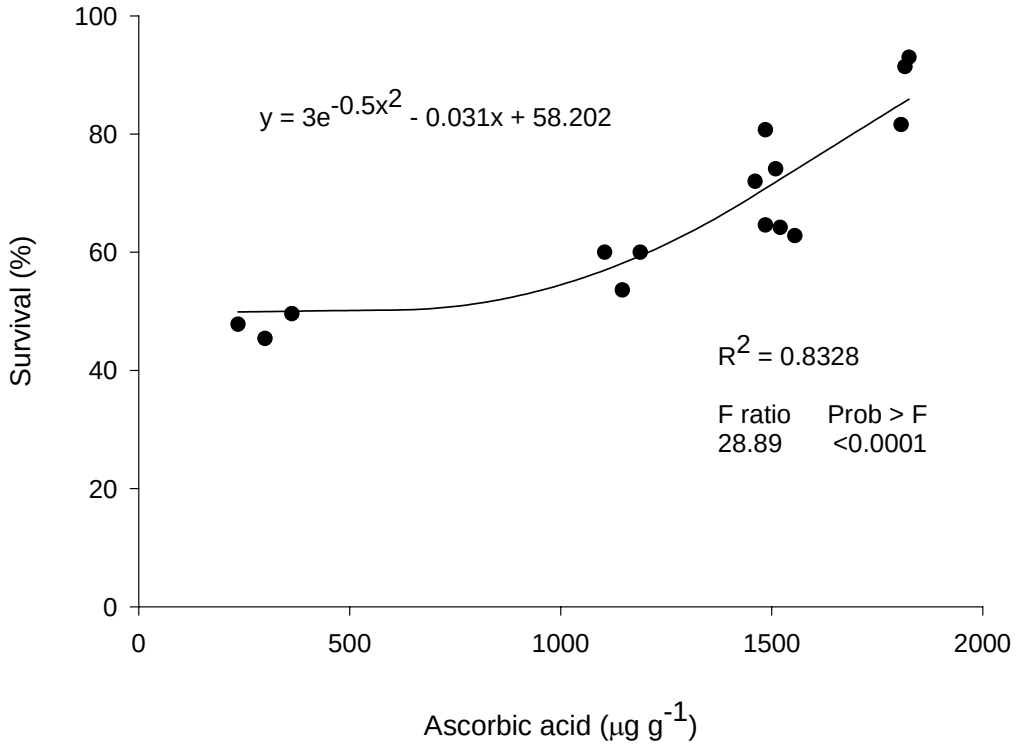

Fig. 8.4 Regression analysis of phyllosoma survival at Stage III and ascorbic acid levels (AA, µg g^{-1} dw) (n=15 phyllosoma groups). For the phyllosoma feed regime, see Fig. 8.1.

8.5.3. Phyllosoma stress indices

Phyllosoma stress indices underwent a number of changes during the culture period from hatch until Stage III (Fig. 8.5). Stage I phyllosoma had a stress index at hatch of 80. This increased 3-fold by Day 3, with a 4-fold increase attained by Day 6 where it was maintained until Day 9. Stress indices in phyllosoma on Days 14 (Stage II) and 28 (Stage III) were reduced to approximately the same levels as attained on Day 3. A number of significant differences were noted between individual dietary groups sampled on the same day (ANOVA, $P< 0.05$). In general, by Day 14 (Stage II) D-0, D-3 and D-6 phyllosoma had lower stress indices, while the stress indices of plain, algal and D-9 phyllosoma were elevated. Of particular

note is the lower stress index exhibited on Day 28 (Stage III) by D-0 phyllosoma compared to all other groups.

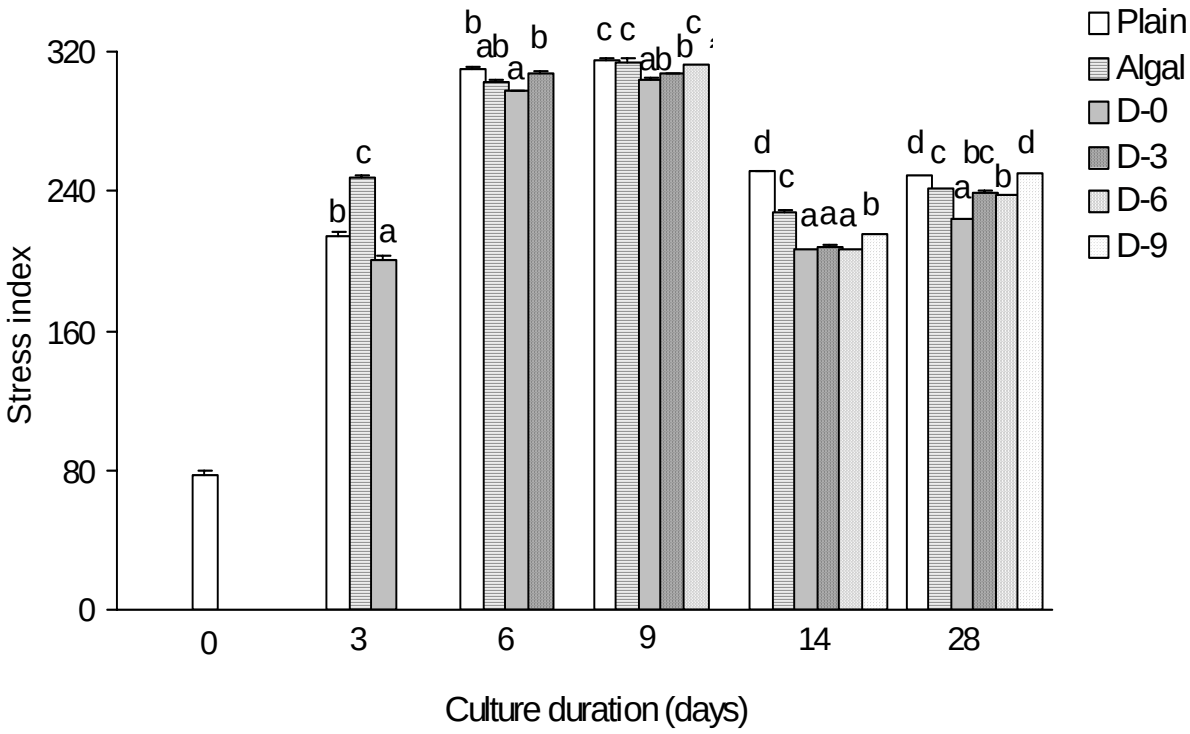

Fig. 8.5 Stress indices at hatch (Day 0) and during culture at Stage I (Days 3, 6 and 9), Stage II (Day 14) and Stage III (Day 28). For the phyllosoma feed regime, see Fig. 8.1. On Days 3 – 9, those feed treatments not shown were yet to commence feeding with ascorbyl-2-phosphate (A2P) enriched *Artemia*, so were equivalent to the plain treatment. Different letters denote a significant difference between phyllosoma survival within a Stage (ANOVA, $P<0.05$). Samples were in triplicate with each replicate consisting of 20 phyllosoma n=3.

There was a significant relationship between the concentration of AA in Stage III phyllosoma and their stress indices (Fig. 8.6). Phyllosoma that had AA tissue concentrations of greater than 1150 μg g^{-1} also had reduced stress indices (valid between the AA values of 300 and 1816 μg g^{-1}). A correlation was evident between the independent factors of larval survival and stress indices (Fig. 8.7), whereby high larval survival was correlated with low stress indices and vice versa.

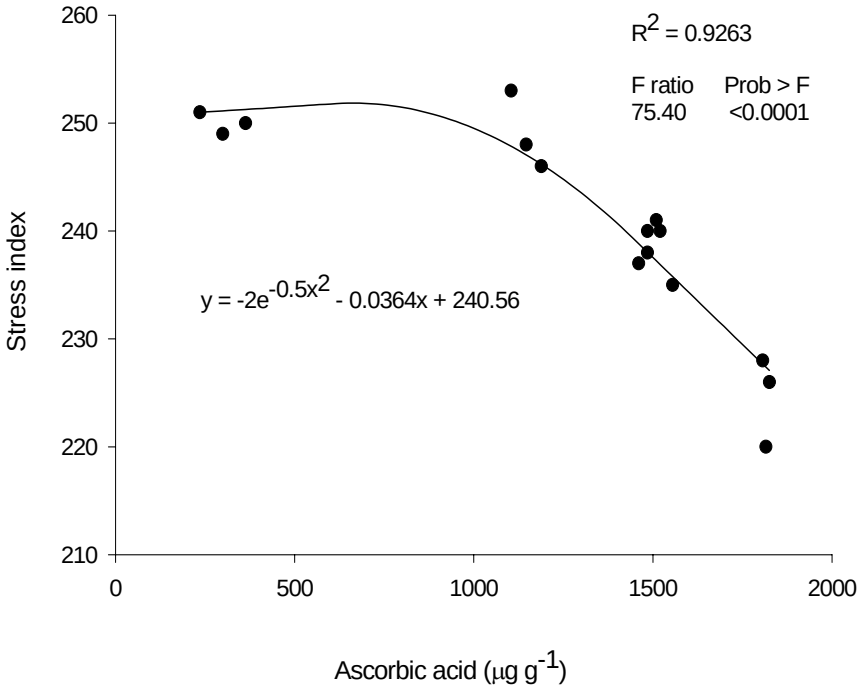

Fig. 8.6 Regression analysis of phyllosoma stress index at Stage III and ascorbic acid levels (AA, μg g^{-1} dw) (n=15 phyllosoma groups). For the phyllosoma feed regime, see Fig. 8.1.

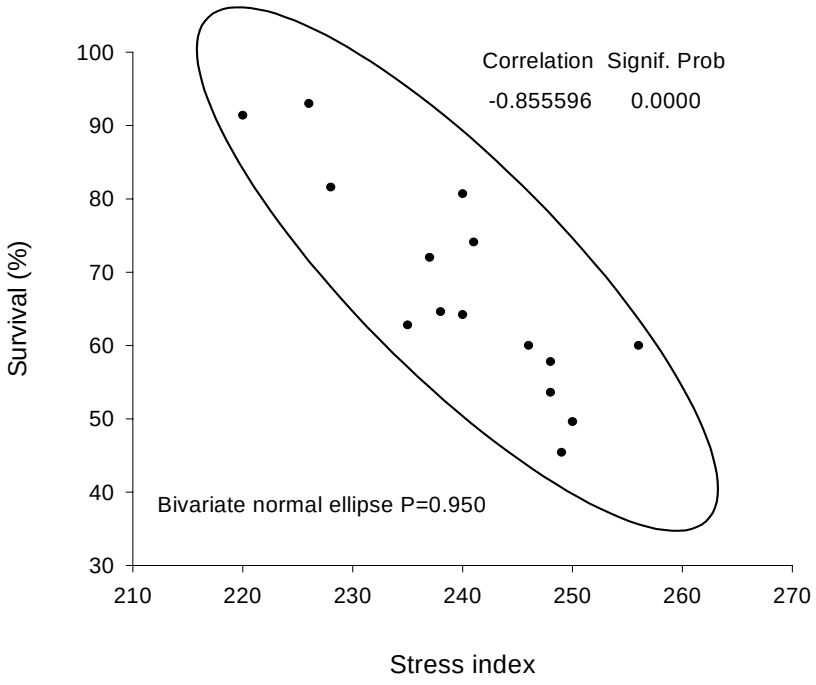

Fig. 8.7 Correlation analysis of survival (%) and stress index at Stage III (n=15 phyllosoma groups). For the phyllosoma feed regime, see Fig. 8.1.

8.6. Discussion

In a number of crustacean and fish species, a link exists between the provision of AA in mega doses and improvements to their normal patterns of growth, survival and stress resistance (Liu et al., 1989; Merchie et al., 1993; Kontara et al., 1997; Merchie et al., 1998; Kolkovski et al., 2000; Lim et al., 2002). The degree of improvement to these parameters has been demonstrated to be species and size specific (He and Lawrence, 1993) and often peculiar to culture conditions (Merchie et al., 1993).

During our study, the largest animals during Stage III were present in the algal, D-0 and D-3 phyllosoma treatments, suggesting that the continuous supply of large doses of AA may be more beneficial to growth than its provision closer to ecdysis. In algal phyllosoma, it is likely that improvements were attributed to a suite of nutritional factors supplied by *Artemia* algal enrichment, possibly including AA. Nelson et al. (2002) noted differential growth in *Artemia* as a result of a range of dietary regimes, with algae providing superior growth to other inert diets. For D-0 and D-3 phyllosoma treatments size benefits were associated with continuous levels of dietary AA as seen in *Clarias gariepinus* and *Penaeus monodon* (Magarelli et al., 1979; Merchie et al., 1993, 1995b; Shiau and Hsu, 1994).

In a number of species, including *Ictalurus punctatus, Stizostedion vitreum, Penaeus vannamei* and *Macrobrachium rosenbergii*, the provision of supplementary AA, had beneficial affects on survival, and in some cases stress resistance (Liu et al., 1989; He and Lawrence, 1993; D'Abramo et al., 1994). This agrees with findings of our study where there was a high correlation between phyllosoma AA concentration, survival and stress indices. One anomaly present in the data was the lower levels of AA present in algal phyllosoma combined with intermediate levels of survival, again suggesting that a suite of nutritional factors contributed to improved survival in this treatment (e.g. high concentration of PUFA, transferred from *C. muelleri*, Nelson et al., 2002).

The link between survival and AA concentrations may be related to the pattern of mortality observed during early larval stages; that is, mortality was confined to the period during and immediately post-ecdysis rather than during the intermoult

interval (personal observation). Ecdysis-related mortality is often associated with unsuccessful attempts by crustaceans to extricate themselves from their old exoskeleton (D'Abramo et al., 1994; Magarelli et al., 1979; Chen and Chang, 1994), a condition exacerbated by inadequate levels of AA for collagen production (Magarelli et al., 1979; Moreau et al., 1998). In part, this may explain why D-0 phyllosoma achieved the highest level of survival surpassing that of other treatments. The reduction in survival in D-3, D-6 and D-9 phyllosoma treatments may be attributed to the delayed start of AA supplementation, which failed to saturate phyllosoma tissues (Dabrowski, 1992b). Plain and algal phyllosoma treatments were supplied with constant, but low concentration of AA, in contrast to D-0 phyllosoma resulting in an apparent dose response reduction in survival. A number of other factors may have had an impact upon AA availability and hence survival during ecdysis, including metabolism without replenishment during the pre-ecdysis non-feeding period and increased stress-related demand for AA associated with ecdysis (Moreau et al., 1998; Merchie et al., 1996). The pattern of survival associated with phyllosoma AA content agrees with the proposal of Dabrowski (1992b) that AA tissue concentration reflects the survival potential of the cultured animal.

As with a number of other fish and crustacean species (Merchie et al., 1997; Dabrowski and Ciereszko, 2001), the specific AA requirement of *J. edwardsii* phyllosoma varies with developmental ontogeny evident by different sequestering, storage and usage patterns displayed during Stages I and II. During Stage I, a total of 3 days or less was required to achieve the same AA phyllosoma concentration across all A2P boosted groups. During Stage II, the pattern of AA accumulation was reduced compared to Stage I and differed across phyllosoma treatments, with none able to attain the same AA concentration as achieved by continuously supplemented D-0 phyllosoma.

The design of the study did not extend to determining whether stress creates an increased AA requirement (Dabrowski, 1992b; Ishibashi et al., 1992). However, we did demonstrate that increasing phyllosoma AA concentration had a moderating effect on stress indices, implying improved stress resistance, comparable to results found in other fish and crustaceans (Merchie et al., 1998;

Kolkovski et al., 2000). As with these other species (Merchie et al., 1998; Kolkovski et al., 2000), a correlation existed between osmotic stress response and survival of congenetics in culture attributable to the accumulation of dietary AA. Moreau et al. (1998) suggested a reduction in collagen production due to AA deficiencies might impede the capacity of crustaceans to replace water with newly-biosynthesized protein, thus altering their rate of intracellular osmotic regulation post moult. This may in part explain why an osmotic based activity test responds to fluctuations in phyllosoma AA tissue levels.

8.7. Conclusion

During this study, we demonstrated that *J. edwardsii* phyllosoma have an initial capacity to assimilate high levels of AA. This capacity is reduced or has increased demand placed upon it with culture to subsequent stages. With increased phyllosoma AA concentration, there were major improvements to survival associated with a moderation of phyllosoma stress indices.

8.8. Acknowledgements

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9. Chapter Nine - General Discussion and Summary

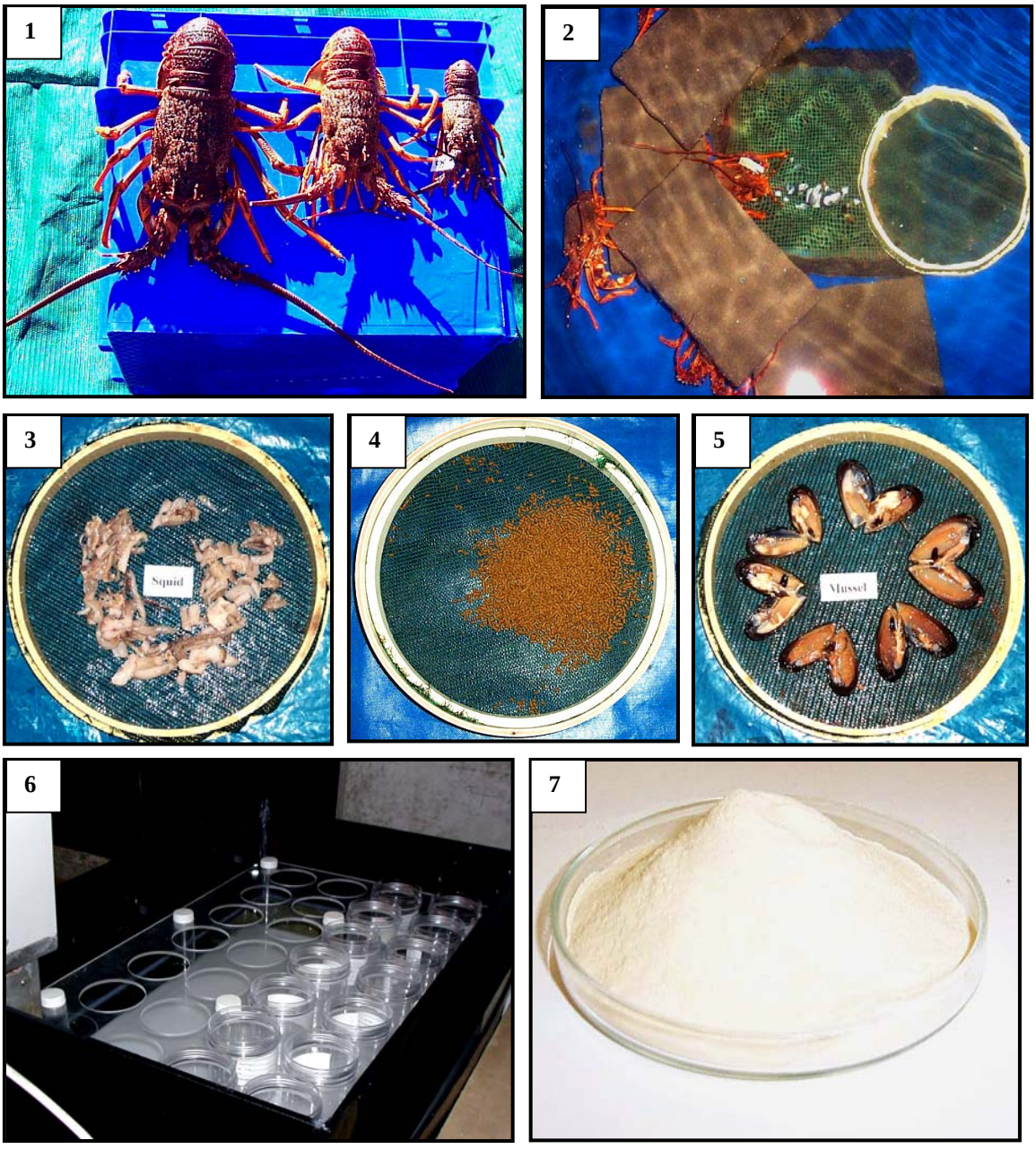

Plate 9. A range of factors found to influence larval competency in the spiny lobster *J. edwardsii.* (1) Large broodstock produce larger more competent larvae. (2) Embryonic development should not be conducted at elevated temperatures. (3, 4, 5) A mixture of fresh and pellet products provide the range of lipids, fatty acids and ascorbic acid required for initial larval competency. (6) Activity tests are a good method to choose the most competent larvae for culture. (7) The provision of supplementary levels of ascorbic acid to newly-hatched phyllosoma through first feeds improves larval survival and growth.

9.1. General Discussion

9.1.1. Activity tests

The development of an activity test that elucidates larval competency provides an important diagnostic tool for use in *J. edwardsii* phyllosoma culture (Chapter 2). The standardized activity test parameters of 21°C at 10‰ provided a short-term response most indicative of survival under current culture conditions. For other crustacean species where activity tests have been developed, they are applied to post-larval or juvenile life-stages in preparation for stocking into grow-out facilities (Tackaert et al., 1989; Dhert et al., 1992; Rees et al., 1994). The activity test developed here allows a pre-emptive view of survival during larval culture and provides information to determine the most competent larvae, ensuring only the best larval groups are selected for culture. While the current activity test for *J. edwardsii* has been devised to assess competence at hatch there is ample evidence to suggest that the predictive capacity of activity tests may be utilized during a number of later developmental stages. Uses may include monitoring general larval health (Dhert et al., 1992; Abi-ayad et al., 1995) or as a barometer to judge competency during specific nutritional, environmental or physiological treatments (Wantanabe et al., 1983; Rees et al., 1994; Kanazawa, 1997; Cavalli et al., 1999). The response of larvae to particular activity test parameters may change with development (Dhert et al., 1992) and require the normal range to be recalibrated or alterations made to the prescribed stress parameters. An insight into the physiological changes that phyllosoma undergo with culture was evident with ascorbic acid (AA) supplementation through to Stage III (Chapter 8). While the current activity parameters were able to differentiate between AA treatment groups, the stress indices were much higher than experienced with newly-hatched larvae.

9.1.2. Environmental parameters

Throughout the course of this study, it was evident that the quality of phyllosoma differed dependant upon various environmental, physiological and nutritional factors primarily resulting from conditions that broodstock were subjected to. One of the early findings demonstrated that while elevated temperatures accelerated embryonic development, reduced the incubation period by almost one-half and increased the duration of larval availability by 2 months (Tong et al., 2000; Smith et al., 2002), this was achieved to the detriment of larval competency (Chapter 2). The definition of larval competency used during this study is based on the survival and growth potential of larvae. This is especially pertinent when selecting *J. edwardsii* larvae for culture, where the larval phase encompasses 11 moult stages, numerous instars and takes in excess of 300 days to complete (Kittaka, 1994). During this study it was found that the use of elevated embryonic temperatures had the adverse effect of producing smaller larvae that are less likely to survive compared to ambient incubated animals. This is a situation that is further exacerbated by culture, as the typical growth pattern for crustaceans is for body size post-ecdysis to be proportional to the size of their previous exoskeleton (Kunish and Anger, 1984; Lovrich and Vinuesa, 1995). In a species with a shorter and less complex life history the impact of size may not be as significant. In their natural environment the odds of *J. edwardsii* larvae surviving are low, with a life-time egg production of 1.6×10^6 (Pollock, 1997) from which only one male and female need to survive to maturity to maintain a stable wild population. To date, poor larval survival has also been demonstrated in captivity and poses a significant impasse to closing the life-cycle of *J. edwardsii*. Therefore, it is essential that efforts to raise larvae are concentrated on producing the largest and most competent specimens. They should not include the use of elevated temperature to artificially reduce the period of embryonic development and as a consequence, produce smaller less competent phyllosoma larvae. The emphasis on the production of out-of-season larvae should instead be focused on phase shifting groups of broodstock to produce larvae at different times of the year under simulated ambient conditions. Such a regime may involve having a group of broodstock 6 months out-of-phase with the normal ambient season. A 6-month

out-of-phase season could be achieved in a controlled environment whereby the changes in water temperature and photoperiod, major cues for maturation in crustaceans, that would occur during a 12-month ambient period (9.5 - 18ºC and 8.5 – 15.3 h daylight, respectively), would be slowed to take 18 months to complete. This would effectively place this group of broodstock 6 months out-of-phase with the ambient season. At this time a 12-month temperature and photo period cycle would be reverted to, albiet 6 months out-of-phase with ambient conditions. Similarly, groups of broodstock may be shifted to 3 and 9 months out-of-phase if desired.

9.1.3. Physiological parameters

There were a number of physiological determinants of larval competency noted during this study (Chapter 3). Predominant among these was establishing that large females produce large competent phyllosoma compared to those from smaller females. Why this occurs is not obvious although theories such as the establishment of a hierarchical system whereby large females have preferential access to food, shelter and males (MacDiarmid, 1989; MacDiarmid and Kittaka, 2000) may contribute to an explanation. As a consequence, a preliminary step to producing viable phyllosoma should include the targeting of large broodstock in preference to smaller animals.

The range of morphological changes that occurred at maturity were similar to those experienced in other spiny lobster species (Kubo, 1938; Gordon, 1960; Berry, 1971), in spite of apparent precocious maturation of females in captivity. This suggests that the alteration of certain morphological traits by *J. edwardsii* at maturity i.e., the elongation of the walking legs in males and the increased length and width of the abdominal segment in females, are secondary sexual characteristics initiated or preceded by maturation cues. Cues that initiate maturation are undefined in this and most crustacean species, however it has been suggested that size at onset of maturity may be influenced by various environmental, social and physiological factors (Annala et al., 1980; Pollock,

1993). This is a fertile area of research worth pursuing for those interested in maintaining somatic growth rather than inducing reproductive development.

The smaller number of viable phyllosoma obtained at hatch compared to the predicted egg number (Chapter 3) raises a number of questions that should be addressed with future studies. The prominent question: Is the large reduction in fecundity representative of what occurs in the wild, i.e., is this normal, or is it the result of hatchery practices?" If the latter is found to be primarily responsible for the reduction in viable phyllosoma at hatch, many of the current animal husbandry processes will need to be examined with a view to minimizing losses and perhaps reducing their impact upon those larvae that are hatching. However, these losses may also occur in the wild, in which case the traditional estimate of fecundity based on egg numbers needs to be reassessed downwards.

9.1.4. Properties of larval food source

While the provision of competent larvae is vital for improving the prospects of large-scale culture of *J. edwardsii* phyllosoma, sustaining them in culture is of equal or greater importance (Chapters 4 and 5). At present, the food source of choice that facilitates growth and survival of phyllosoma is 1.5 mm juvenile *Artemia*, fed at 3 ml^{-1} (Tong et al., 1997; Moss et al., 1999; Ritar et al., 1993). The culture of *J. edwardsii* through to pueruli has been possible using *Artemia* as the sole food source or in combination with other fresh products, most notably mussel gonad (Kittaka et al., 1988; Booth, 1996). While the examination of phyllosoma mouthpart and foregut ontogeny has provided valuable information on the type and texture of preferred larval foods i.e., soft and gelatinous (Johnston and Ritar, 2001), this is a food texture that is at odds with the use of *Artemia* as a food source. As such there is a concerted effort being directed towards the development of an artificial diet (B. Crear pers. comm.). Although recent trials of some of these formulations have demonstrated an unsuitability for early stage phyllosoma due to problems with diet acceptance, physical fouling and associated elevation in the concentration of pathogenic bacteria (A. Tolomei pers. comm.).

At least for the present, the optimal food for *J. edwardsii* phyllosoma and early stage phyllosoma in particular (i.e., up to Stage IV), is juvenile *Artemia*. This is an

Artemia life-stage about which very little is known as most nutritional studies have concentrated on newly-hatched or Day 2 metanauplii (Watanabe et al., 1982; Rees et al., 1994; Rasowo et al., 1995; Narciso et al., 1999). During this study, the uptake of the essential fatty acids (EFA) 20:4n-6 (arachidonic acid, ARA), 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) were examined in metanauplii and juvenile *Artemia* and found to be assimilated with a similar capacity at both life-stages. The EFA, ARA and EPA were assimilated proportional to their inclusion in the enrichment, while DHA was sequestered or perhaps maintained to a lesser degree (Chapter 4). This demonstrates that juvenile *Artemia* can be used with similar effectiveness as metanauplii in the delivery of EFA. An interesting observation was that in the absence of feeding stimuli, the gut was not evacuated, and did not contribute appreciably to the total lipid content of the *Artemia* after 3 h starvation. This is testament to the rapid assimilation of lipid from the diet into body tissue. Concerns over significant lipid losses from the gut of *Artemia* due to the tearing feeding behavior of many larval crustaceans (Abrunhosa et al., 1997; Crain, 1999) are negated with this finding.

The desire to supplement AA as part of a dietary component is in response to improvements to larval competency evident in other species receiving AA boosted *Artemia* metanauplii (Merchie et al., 1995 a,b,c; Merchie et al., 1997). Prior studies have demonstrated that established enrichment protocols using AA emulsions are unsatisfactory for juvenile *Artemia* and unlikely to elicit a competency response (Lim et al., 2002). During this study, the development of a protocol to use a particulate form of AA to enrich juvenile *Artemia* provided a powerful tool for its delivery via a larger prey item (Chapter 5). It was found that AA was maintained in juvenile *Artemia* at high concentrations for 24 h, simulating typical feed presentation periods in temperatures applicable to temperate and tropical species and suggests the ability of juvenile *Artemia* to deliver a number of therapeutic products to larval predator species. This includes the possibility of the delivery of a particulate form of α-tocopherol, a complementary product to AA in the enhancement of larval competency (Cahu et al. 1995).

9.1.5. Nutritional parameters

Biochemical determinants of larval competency were examined primarily from the perspective of manipulating broodstock lipid and AA dietary regimes (Chapters 6 and 7). It was noted that the lipid and fatty acid profiles of broodstock ovary and tail muscle were stable over time despite the provision of an unusual dietary mix of EFA (Chapter 6). This stability extended to the phyllosoma with minimal differences between the fatty acid profiles of animals from two widely differing diets, suggesting that larval profiles are maintained under situations of extreme nutritional duress, as sometimes reported in wild populations (Jernakoff et al. 1993; Barkai et al. 1996; Cox et al. 1997). There was no direct link between fatty acid profiles and survival of larvae in culture or activity tests. Therefore, the stability of both broodstock and phyllosoma lipid and fatty acid profiles and the ability of animals to sequester their requirements from meager supplies should largely negate concerns about the need to supplement broodstock dietary fatty acids to maintain larval competency. The other organ examined was the digestive gland, which demonstrated a large storage capacity for dietary lipids and fatty acids, with no evidence for direct sequestering of these stores during ovarian maturation. Lipid catabolism appeared to occur during periods of inadequate nutrition, which is useful for wild animals experiencing dietary deficiencies. The digestive gland fatty acid profile was malleable and assumed the fatty acid profile of the diet, which may be a potential correlate for identifying those feed sources associated with improved larval competency in wild animals.

The concentration of dietary AA required to saturate broodstock ovaries in *J. edwardsii* was relatively low and concomitant with low ovarian saturation concentrations compared to some other crustaceans (Cavalli, 2000; Wouters et al., 2001). Further, there were minimal benefits to AA supplementation above and beyond that supplied by the basal mix of fresh and compound penaeid diet. Even though AA supplementation prevented the depletion of muscle AA this did not confer any benefit to these animals, with no significant differences noted across the range of broodstock and phyllosoma parameters measured. These results contrast with those in other studies where dietary AA supplementation of broodstock prior to and during ovarian improved fecundity, hatchability and larval

quality (Kontara et al., 1997; Moreau et al., 1998). A combination of fresh and compound diet, as supplied during this study, should provide adequate concentrations of AA to meet the needs of *J. edwardsii* broodstock and embryo during development to larvae.

In contrast, there were a number of tangible benefits obtained with AA dietary supplementation to phyllosoma, including increases to phyllosoma growth and most notably survival. This is the first time that supplementation has shown clear improvements in these parameters beyond that of the standard *Artemia* diet. Previous nutritional studies with *J. edwardsii* phyllosoma have concentrated on manipulating lipid and fatty acid nutritional profiles without any obvious benefits to growth and survival (A. Ritar and P. Hart pers. comm.). It was also noted during this study that phyllosoma supplied with algal enriched *Artemia* were also conferred with considerable improvements to growth and survival compared with unsupplemented *Artemia*. The reasons for this are unknown but are likely to be due to the provision of a suite of nutritional factors that are missing or lacking in existing larval foods. This may be an indication that a range of natural marine dietary products may be of benefit to furthering larval culture in this species. An important proposition was confirmed during this study, namely that the larval tissue concentration of AA reflects an animals survival potential (Dabrowski, 1992) i.e., the ability to increase the amount of AA in early stage phyllosoma was correlated with an improvement to survival. However, this proposition did not extend to broodstock or developing embryo (Chapter 7) and may decrease with subsequent stages of phyllosoma development, but this is a future area of research.

9.2. Summary

This study developed a robust technique to assess larval competency at hatch providing the tools to circumvent problems associated with larval competency at the commencement of culture. A capacity to extend the analysis of competency to later developmental stages was also demonstrated. The boundaries of *Artemia* live feed protocols and regimes were extended providing a better understanding of the requirements and capacity of this important aquaculture feed source. A range of

environmental (temperature manipulation), physiological (broodstock size, larval size) and nutritional (AA) factors were identified which will assist in the selection of larvae of optimal competency. To further studies of phyllosoma culture a focus upon ascertaining the physiological causes of mortality will be required, the prime suspect being bacterial contamination, influenced by factors such as feed composition and delivery, water quality and tank configuration. These issues are problematic in this species due to the prolonged larval duration and the effect that they may have upon even competent larvae if exposed for sufficient duration.

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